Studies on a new Human Herpesvirus, Kaposi's sarcoma-associated Herpesvirus

By
Bianca Ariane Elzinger

Department of Virology
Royal Free and University College Medical School
London
United Kingdom
W1P 6DB

This thesis is submitted to the University of London
in fulfillment for the degree of
Doctor of Philosphy (Ph.D.)
2000

Dedication

To my parents

Die Dinge, die uns geschehen, geschehen nie umsonst, nur um ihrere selbst willen, jede Begegnung, jedes kleine Ereignis birgt eine Bedeutung in sich, das Verstaendnis fuer sich selbst entsteht aus der Breitschaft, diese anzuerkennen, aus der Faehigkeit, in jedem beliebigen Moment die Richtung zu aendern, aus der alten Haut zu schluepfen wie eine Eidechse beim Wechsel der Jahreszeiten.

Susanna Tamaro

ABSTRACT

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), also called human herpesvirus 8 (HHV8), has been been identified in all epidemiological forms of KS as well as in tissue obtained from primary effusion lymphoma (PEL) and Multicentric Castleman's disease (MCD). The KSHV genome contains several putative oncogenes, suggesting that viral infection may induce cellular transformation and tumorgenesis.

Herpesviruses encode a number of different surface glycoproteins, which are involved in virus-host interactions. Studies have shown that the viral glycoproteins H and L form a complex that plays an essential role in viral attachment and cell to cell fusion. Both glycoproteins have been identified in KSHV and were expressed in mammalian cells. Expression studies revealed that KSHV gH and gL exhibit similar features to those seen in other herpesviruses. However, KSHV gL appears to traffic independently and may function in cell to cell fusion processes even when expressed alone.

KSHV *de novo* infections are rare and the lack of a reliable cell culture system has delayed pathogenesis studies. As part of this thesis the hepatoma cell line HepG2 has been shown to allow limited KSHV infection, as judged by nested PCR. Studies have shown that infection leads to increased apoptosis, although viral replication could not be detected. Furthermore, Epstein Barr Virus (EBV) appeared to modulate the ability of KSHV to infect HepG2 cells.

Finally, a microtitre plate assay has been established for the quantification of the KSHV genome. A comparison of plasma and serum samples obtained at the same time point showed that plasma is more reliable in testing for KSHV, the DNA copy number in serum samples being reduced up to 10 fold. In conclusion, this new assay is a potentially useful tool for both diagnostic proposes and research studies.

Acknowledgments

I would like to thank everybody in the Department of Virology for their continuing support, advice and encouragement during this project, especially within the last couple of months. I think without this I would never have been able to board this plane.

Also, a big thank you goes to my supervisor, Dr Peter Balfe, who had to keep correcting my 'Germanic' sentences over the past few months.

Furthermore, I wish to say thank you to Dr Nicola Brink and Professor Richard Tedder for organizing all the clinically relevant samples, without these studies would not have been possible.

I am also very thankful to Anna Hayman, Samreen Ijaz and Emma Baker who had to listen to all my complaints. I hope you will come to visit me soon. Thanks to Dave Bibby to use his computer more or less constantly.

A special thanks to someone who is far away but kept saying the right things!

Finally, I would like to thank my parents for their continuous support while I was in London, it helped me immensely.

This PhD studentship was funded by the Middlesex Special Trustees.

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Common Abbreviations

AIDS	Acquired Immune deficiency Syndrome
Amp	ampicillin
bp	base pair
CMV	cytomegalovirus
DISC	death inducing signalling complex
DNA	deoxy-ribonucleic acid
dNTP	2' deoxyribonuctleotide 5' triphosphate
E.coli	Escherchia coli
EBV	Epstein-Barr Virus
EDTA	ethylene-diaminetetra-acetic acid
EIA	enzyme-linked immunosorbent assay
FACS	fluorescent activated cell scanner
FITC	fluorescein-5-isocyanate
FLICE	Fas-associated death domain protein-like IL-1-converting enzyme
g, mg, μg, ng	gram, milligram, microgram, nanogram
gH	glycoprotein H
gL	glycoprotein L
HHV8	Human herpesvirus 8
HIV	Human Immunodeficiency Virus
HRP	Horse Radish Peroxidase
HSV	Herpes simplex virus
HVS	herpesvirus saimiri
ICE	interleukin-1beta converting enzyme
IFA	indirect immunofluorescence
IL-6	interleukin 6
IN	insoluble
kDa	kilo Dalton
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
LY	lysate
M, mM, μM, nM	molar, millimolar, micromolar, nanomolar
mA	milliampere
mAb, MAb	monoclonal antibody
MCD	Multicentric Castleman's disease
ml, μl	milliliter, microliter
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEL	Primary effusion lymphoma
RNA	ribonucleic acid
S	supernatant
SDS	sodium dodecylsulphate

SOL	soluble
TAE	Tris-acetate EDTA buffer
TE	Tris-HCl EDTA
Tris	tris (hydroxymethyl) aminomethane
V	Volt
v-cyc	viral cyclin
vFLIP	FLICE inhibitory proteins
vGPCR	viral G protein coupled receptor
vIL8R	viral interleukin 8 receptor
vMIP	viral macrophage
VZV	Varicella zoster Virus
X-Gal	5-bromo-4-chloro-3-indoyl-D0galatoside

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Chapter 5

1. Introduction

Kaposi's sarcoma was originally described in 1872 by the dermatologist Moritz Kaposi as a multiple pigmented sarcoma affecting elderly Mediterranean men (Kaposi, 1872). To date several epidemiological forms have been classified. In recent years the general prevalence of this vascular tumour in the USA and UK was low, at 0.05 per 100 000 of the population (Biggar *et al.* 1984). However, with the emergence of the HIV epidemic, the incidence of KS increased. In 1990, approximately 30% of HIV infected gay men suffered from KS during their illness (Jacobson *et al.* 1990).

Kaposi sarcoma lesions are histologically complex and evolve over time, from a patch or plaque-like lesion at early stages to nodular lesions characteristic of disease progression. Several hypotheses regarding the pathogenesis of this vascular tumour have been suggested, including both infectious agents and cytokine mediated processes. In 1994 a study by Chang and colleagues found a novel herpesvirus present in KS tissue (Chang *et al.* 1994). Since its discovery this virus, now known as KS associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8), has been the focus of many studies regarding its role in the pathogenesis of Kaposi's sarcoma.

1.1 Kaposi's Sarcoma (KS)

1.1.1 Epidemiological forms of Kaposi's sarcoma

Kaposi's sarcoma is a multifocal vascular neoplasm involving the skin, visceral organs and lymph nodes. The lesions are complex and contain distinctive proliferating spindle cells, activated endothelial cells and infiltrating inflammatory

cells (Roth *et al.* 1992). The classic form of KS predominantly affects elderly male patients of southern European heritage (Franceschi and Geddes, 1995). The tumour is also frequently described in patients from Israel, mainly in the Jewish population (Iscovich *et al.* 1998b; Iscovich *et al.* 1998a) and in other Middle eastern countries. Disease progression in classic KS patients occurs over a period of several years. In patients suffering other forms of KS, including endemic KS of HIV negative individuals in central regions of Africa, iatrogenic KS, and AIDS-associated KS, the disease is more aggressive. In HIV infected individuals the underlying immunsuppression leads to a fulminant disease starting with skin lesions and often affecting systemic organs (lung, liver, gut spleen) (Beral, 1991a; Beral, 1991b). KS is the most common AIDS-associated malignancy and has been a leading cause of morbidity among AIDS patients (Beral *et al.* 1990).

The endemic form of KS can be found in some equatorial countries of Africa. Here, KS is described in both young and elderly HIV-negative patients. Before the HIV epidemic KS was common in some areas of Sub Saharan Africa. With the advent of AIDS a 20 fold increase in the incidence of KS in Uganda and Zimbabwe was observed (Cook Mozaffari *et al.* 1998; Chang *et al.* 1996b) and KS has become one of the leading malignancies in sub-Saharan Africa. Disease progression is more aggressive than for classical KS, in particular for children where lymph node involvement is common. Endemic KS leads to the majority of death in young patients (Ziegler and Katongole Mbidde, 1996).

Finally, the incidence of this vascular tumour has increased among renal transplant recipients and other patients receiving immunosupressive therapy. This form is known as post-transplant KS or iatrogenic KS (Beral, 1991a).

1.1.2 Histology of KS

Kaposi's sarcoma is characterised by bundles of spindle-shaped cells and endothelial-lined vascular spaces with lymphocytic infiltrates. Commonly red blood cells percolate in the spaces between the spindle cells (Cockerell, 1991). These spindle cells produce pro-inflammatory and angiogenic factors that are likely to play an important role in the recruitment of additional cells to the sarcoma (Ensoli *et al.* 1991). Also, KS cells are highly dependent on inflammatory cytokines in culture. This observation led to the hypothesis that KS pathogenesis may be influenced by inflammatory cytokines and angiogenic factors (Nakamura *et al.* 1988; Ensoli *et al.* 1992).

KS is not a typical sarcoma, in that multiple lesions may appear simultaneously at several locations, but may disappear completely over time. As mentioned earlier KS starts as a patch or plaque-like lesion at early stages and develops into the nodular lesions characteristic of later stage disease. In early stages small irregular endothelial-lined spaces, surrounded by normal blood vessels and inflammatory infiltrates of lymphocytes can be detected. As the disease progresses the number of spindle shaped cells increase throughout the dermis and form slit like vascular channels containing erythrocytes (patch stage). In the final, nodular stage, the lesions are characterised by sheets of spindle cells.

KS cells represent a heterogeneous population, as indicated by the variety of surface markers. The cells identified by histology are mainly activated endothelial cells mixed with fibroblasts, smooth muscle cells and cells of dentritic and monocytic origins (Regezi *et al.* 1993; Roth *et al.* 1992). The clonality of KS is frequently discussed, as the exact tumor cell type is controversial. Especially in early lesions spindle cells are a minority. Spindle cells are usually not prominent until late stage disease. Recent studies have demonstrated that spindle cells belong to an endothelium lineage that expresses the vascular endothelium growth factor receptor-3 (VEGFR-3), a marker of lymphatic vessels (Dupin *et al.* 1999). Other studies analysing inactivation pattern of the X chromosome, a marker for clonality, revealed both a monoclonal and polyclonal pattern (Rabkin *et al.* 1995; Delabesse *et al.* 1997). KS may be initially a non-clonal proliferation, but may sometimes develop into a clonal malignancy with metastases during advanced disease (Rabkin *et al.* 1997).

1.1.3 Pathogenesis of KS, chemokines and viruses

Kaposi's sarcoma lesions produce high levels of cytokines both in vivo and in culture. Several cytokines and growth factors have been implicated in the proliferation of KS spindle cells, including oncostatin M (Miles *et al.* 1992), platelet derived growth factor (PDGF) , Interleukin 1β (IL-1β) (Sturzl *et al.* 1995), Interleukin 6 (IL-6) (Miles *et al.* 1990), Interleukin 8 (IL-8) (Sciacca *et al.* 1994), basic fibroblasts growth factor (bFGF) (Ensoli *et al.* 1994a; Ensoli *et al.* 1994b), vascular endothelial growth factor (VEGF) (Cornali *et al.* 1996), macrophage

chemotactic protein-1 (MCP-1) (Barillari *et al.* 1992), and the Human Immunodeficiency Virus-1 (HIV-1) Tat protein (Ensoli *et al.* 1990). Data from gene expression studies further support their involvement in KS pathogenesis as angiogenic properties of IL-6, bFGF and IFN gamma could be demonstrated in vitro (Sciacca *et al.* 1994; Cornali *et al.* 1996). In particular IL-6 has been shown to enhance proliferation of KS cells in culture (Miles *et al.* 1990). As disease progression in HIV-associated KS is more aggressive, it has been suggested that proteins encoded by HIV may influence tumour growth. Observations showed that the transcription activator protein, Tat, in combination with bFGF, stimulate the proliferation of KS associated spindle cells *in vitro* and can induce KS-like lesions in mice (Ensoli *et al.* 1994b). This HIV encoded protein also induces cellular genes that are pro-proliferative and pro-inflammatory (MCP-1, IL-6) in KS and possibly enhance the recruitment of leukocytes to KS lesions (Kelly *et al.* 1998)).

While *in vitro* and animal models strongly suggested that cytokines and growth factors play an important role in the development of KS (Ensoli *et al.* 1992), epidemiological data implicated a sexually transmitted cofactor (Beral *et al.* 1990). AIDS patients with Kaposi's sarcoma are predominantly gay or bisexual men, rather than patients who acquired the virus through heterosexual contact, haemophiliacs or intravenous drug users (Beral, 1991b). These findings favour an infectious agent or co-factor in the development of KS. Infectious agents such as cytomegalovirus (CMV) (Giraldo *et al.* 1972) human herpesvirus 6 (HHV-6), human papilloma virus (HPV), BK virus and other viral or bacterial pathogens have all been linked to KS (Monini *et al.* 1996b; Wang *et al.* 1993). However, in

1994 a research group in New York detected herpesvirus-like DNA from biopsies of AIDS-associated KS using a subtractive DNA hybridisation technique known as representational difference analysis (RDA) (Lisitsyn, 1995; Chang *et al.* 1994). The technique relies on cycles of subtractive hybridisation and polymerase chain reaction to enrich and isolate nucleotide sequences which differ between KS affected tissue and unaffected surrounding cells. Two subgenomic BamH1 fragments of DNA, designated $KS330_{Bam}$ and $KS631_{Bam}$, were isolated. Subsequent computer-assisted homology searches revealed that these novel sequences were related to the minor capsid and tegument protein genes of the lymphotropic γ-herpesviruses Epstein Barr (EBV) and Herpesvirus saimiri (HVS) (Chang *et al.* 1994, Albrecht *et al.* 1992; Baer *et al.* 1984). More recently, similarities between this newly identified herpesvirus and the rhesus monkey rhadinovirus (RRV) (Searles *et al.* 1999; Desrosiers *et al.* 1997) as well as macaque retroperitoneal fibromatosis virus isolates Mn and Mm (Rose *et al.* 1997) have also been described. Further characterisation of this newly identified agent, named Kaposi's sarcoma-associated herpesvirus or human herpesvirus 8, confirmed its classification as a member of the gamma-2 herpesvirus family (genus *Rhadinovirus*) (Moore *et al.* 1996b). Visualization of herpesvirus like particles by electron microscopy provided additional evidence for the viral origin of these sequences (Said *et al.* 1996b).

1.2 Family of Herpesviridae

Herpesviruses have the capacity to establish latent infections in their host, with the genome persisting in the nucleus. Hence these viruses are frequently reactivated in AIDS patients and following immunosupressive therapy for organ transplantations or cancer.

The *Herpesviridae* are a family of enveloped virions, approximately 120-200nm in diameter, comprising an inner core that is surrounded by an icosahedral capsid, and a lipid-bilayer, the envelope. The number of glycoproteins embedded in this viral envelope varies for each herpesvirus, for example HSV encodes at least 11 glycoproteins.

The herpesvirus genome is a linear double stranded DNA molecule, which circularises upon release from capsids into the nuclei of infected host cells (Furlong *et al.* 1972). During latency only a small subset of genes is expressed to maintain the viral genome and maintain a persistent infection (Croen, 1991). Herpesviruses encode a large number of enzymes involved in nucleic acid metabolism, DNA synthesis and the processing of proteins. Many of these genes are not essential for viral replication and presumably encode other functions, such as cellular growth control, pathogenicity, supression of the host immune response or maintenance of viral latency (McGeoch, 1989). Viral gene expression is both highly complex and tightly regulated, with sequential transcription and translation of immediate early, early and late genes (Roizman, 1993). One may generalize that viral immediate early genes are responsible for the activation of lytic genes and that early synthesised proteins regulate the transcription of later genes (Chevallier Greco *et al.* 1986; Countryman and Miller, 1985; Nicholas *et al.* 1991;

Whitehouse *et al.* 1998). During lytic infection the virus induces the shut down of host cell protein and nucleic acid synthesis to produce its own progeny virions. Usually herpesviruses have characteristic cytopathic effects (CPE), such us the formation of multinucleated giant cells (VZV, HCMV), swollen, rounded cells (HSV), or intranuclear inclusion bodies (HSV, HCMV) (White and Fenner, 1994). The classification of the herpesviridae into three subfamilies was based originally on these biological properties. However, with the increasing number of sequences available from each herpesvirus the taxonomy will in future be based more on DNA sequence similarity, genome arrangements and the conservation of particular genes.

1.2.1 Alphaherpesvirinae

This subfamily includes the herpes simplex viruses 1 and 2 (HSV-1; HSV-2) and varicella-zoster virus (VZV). Recurrent HSV-1 infection is generally associated with labial herpes, whereas HSV-2 is mainly responsible for genital herpes. VZV causes chickenpox during childhood and herpes zoster (shingles) on reactivation in adults.

These viruses have a variable host range and a relatively short reproductive cycle. In culture viral growth is rapid and leads to destruction of the infected cell. Latency is primarily established in sensory neurons.

1.2.2 Betaherpesvirinae

The human cytomegalovirus (HCMV), human herpesvirus 6 (HHV6) and human herpesvirus 7 (HHV7) are members of the beta herpesvirinae subfamily. CMV infection is acquired subclinically during childhood but can cause severe damage in immunocompromised adults. Also, during pregnancy primary CMV infection can lead to severe congenital abnormalities in the fetus. Reactivation of latent CMV infection is one of the most common opportunistic infection in HIV-infected patients and can lead to death.

HHV6 and HHV7 infect children in the first two years of life and are associated with exanthem subitum (Levy, 1997). An association of HHV6 with encephalopathy have been suggested but this is still under investigation. Biologically, betaherpesvirinae show a long reproductive cycle *in vitro* and produce large, often multinucleated, cells (cytomegalia). Latency is established in secretory glands, lymphoreticular cells, the kidney and other tissues.

1.2.3 Gammaherpesvirinae

Herpesviruses of the subfamily gammaherpesvirinae are involved in the pathogenesis of several lymphomas or carcinomas. Representatives of this group exhibit oncogenic potential and are associated with B- and T-cell lymphomas. Viruses replicate and persist in lymphoblastoid cells (Roizman, 1993). Members include the Epstein Barr virus (EBV), the squirrel monkey virus, herpesvirus saimiri (HVS) and others. On the basis of sequence similarity, the newly identified herpesvirus KSHV has been classified as a gammaherpesvirus, most closely related to the gamma-2-herpesviruses. Other gammaherpesvirus subgroup with

sequence similarities to KSHV are the murine herpesvirus 68 (MHV68) and the recently identified retroperitoneal fibrosis herpesvirus (RPHV). Both viruses are capable of inducing tumours in animals. Table 1.1 lists the known gamma herpesviruses that are related to KSHV.

Virus	Host	Associated tumor
Epstein Barr Virus (EBV)	Humans	Burkitt's lymphoma Hodgkin's disease Nasopharyngeal carcinoma Post-transplant lymphoproliferations
Herpesvirus saimiri (HVS)	New World monkeys	T cell lymphoproliferations
retroperitoneal fibrosis herpesvirus (RPHV)	Old World monkeys	Retroperitoneal fibrosis
Murine herpesvirus 68 (MHV68)	Mice	Lymphoproliferations

Table 1.1: Gammaherpesviruses that are related to the newly identified Kaposi's sarcoma associated herpesvirus (KSHV)

Epstein Barr Virus causes infectious mononucleosis, in certain geographic areas the virus has been linked to a number of malignant lymphomas, including Burkitt's lymphoma (BL) in Sub Sahara Africa and naso-pharyngeal carcinoma (NPC) in East Asia (Kieff, 1996). *In vivo* EBV is able to infect B lymphocytes as well as epithelial cells. *In vitro* EBV infection is largely restricted to primate B lymphocytes; epithelial cells can be infected but infection is abortive and the virus is lost on passage. Most knowledge of EBV pathogenesis is based on culture

systems using B lymphocytes, where the virus transforms resting B lymphocytes to proliferate indefinitely in culture (for review see Kieff, 1996). These lymphoblastoid cell lines (LCL) are similar to antigen-driven B cells and are used successfully for the production of human monoclonal antibodies (Gorny *et al.* 1998; Liu *et al.* 1993). For example, PBMCs derived from HIV-infected individuals can be transformed with EBV *in vitro*. The resulting LCLs are then screened for antibody production and, to maintain them *in vitro* fused to a mouse myeloma cell line (Gorny *et al.* 1998). Fusion with a second LCL is a second popular method of immortalization (Foung *et al.* 1984).

The latency pattern of LCL has been described, so far 11 viral genes have been identified which play a role in persistent infection and transformation: two small non-polyadenylated RNAs (EBER1; EBER2); six nuclear antigens (EBNA 1, 2, 3A, 3B, 3C, LP) and three integral membrane proteins (LMP1, 2A, 2B) (Kieff, 1996). Studies with recombinant viruses showed that EBNA2, EBNA3A, EBNA3C and LMP1 are essential for the immortalization process (Cohen and Kieff, 1991; Kaye *et al.* 1996). The EBNA1 gene is involved in episome maintenance. In addition the EBNA2 gene determines differences between EBV type 1 and type 2 in transforming B cells (Kieff, 1996; Hammerschmidt and Sugden, 1988). A more detailed description of these EBV gene products is given later in the chapter with the appropriate KSHV counterpart.

Herpesvirus saimiri (HVS) is the prototype of rhadinoviruses, the second subgroup of the gammherpesvirinae. This virus is found in the peripheral blood mononuclear cells of healthy squirrel monkeys. HVS is apathogenic in its natural

host but can cause fulminant T cell lymphomas in other species, including New World monkeys (Fleckenstein *et al.* 1982). Furthermore, HVS can immortalize peripheral blood lymphocytes of both simian and human origin (Biesinger *et al.* 1992). Some human CTL lines have been established by infecting human T cells with HVS (Berend *et al.* 1993; Lacey *et al.* 1998) A number of genes of HVS are not found generally among the herpesvirus family. These genes exhibit significant homology with cellular proteins, including complement control proteins, cyclins and seven transmembrane G protein coupled receptors (Albrecht *et al.* 1992). The newly identified herpesvirus KSHV is a close relative of HVS that infects humans (Chang *et al.* 1994). Its genome contains all the open reading frames that are conserved throughout the herpesvirus family together with a high number of unique genes that may be important in angiogenesis, cellular growth control and regulating antiviral immunity (Russo *et al.* 1996).

1.3 Kaposi's sarcoma associated herpesvirus (KSHV)

1.3.1 Detection of the KSHV genome in KS

Since its first description in 1994, KSHV/HHV8 DNA sequences have been detected by polymerase chain reaction in all epidemiological forms of the disease, in both fresh biopsies and in the vast majority of paraffin embedded material (Boshoff *et al.* 1995; Moore and Chang, 1995; Schalling *et al.* 1995; Dupin *et al.* 1995; Ambroziak *et al.* 1995; Whitby *et al.* 1995). By using in-situ PCR KSHV can be detected in all stages of the disease from early patches to nodular lesions (Kennedy *et al.* 1998; Dupin *et al.* 1999). Using a monoclonal antibody specific for the KSHV nuclear antigen (anti-LANA), only 10% of cells in early KS lesions harbor the virus (Dupin *et al.* 1999). The amount of cells staining positive for LANA increases to 90% as disease progresses. These findings support a direct role for the virus in the etiopathogenesis of KS (Dupin *et al.* 1999). In KS lesions KSHV sequences have been localized to spindle and endothelial cells and to monocytes. Prior to extensive spindle cell formation herpesviral sequences are limited to the flat endothelial cells of the lesion (Boshoff *et al.* 1995; Staskus *et al.* 1997; Sturzl *et al.* 1997; Rainbow *et al.* 1997). The viral genome is maintained as an episome and at least one copy per cell can be detected (Decker *et al.* 1996; Chang *et al.* 1994). The majority of spindle cells seem to be latently infected, with a sub population supporting lytic replication (Davis *et al.* 1997; Staskus *et al.* 1997; Orenstein *et al.* 1997; Rainbow *et al.* 1997; Zhong *et al.* 1996). Several attempts to establish an *in vitro* culture system for KSHV using spindle cells isolated from KS have been unsuccessful. Spindle cells have a limited life in tissue culture and even well established KS cells lose the viral genome upon

passage (Aluigi *et al.* 1996; Ambroziak *et al.* 1995).Also non-involved tissue from KS patients, including skin, lymphoid tissue, prostate, semen, saliva and PBMC, has been shown to harbor KSHV DNA (Harrington, Jr. *et al.* 1996; Koelle *et al.* 1997; Mesri *et al.* 1996; Rainbow *et al.* 1997; Staskus *et al.* 1997; Monini *et al.* 1996a; Dupin *et al.* 1995; Howard *et al.* 1997). Furthermore, in-situ hybridization to detect the minor capsid protein mRNA has shown that monocytic cells are productively infected with KSHV. However, not all monocytes stained positive and therefore transmission of virus from spindle cells to monocytes was thought to be rather unlikely (Blasig *et al.* 1997). It has also been demonstrated that the virus replicates in PBMCs of KS patients (Decker *et al.* 1996). PCR studies of PBMCs from healthy individuals have found the virus to be uncommon (Gao *et al.* 1996b; Kedes *et al.* 1996). In contrast in areas at high risk for KS, KSHV has been detected at a high frequency (Sirianni *et al.* 1997). It has also been demonstrated that the detection of KSHV in the peripheral blood of HIV infected patients precedes KS development and is strongly correlated with increased KS risk (Gao *et al.* 1996a; Whitby *et al.* 1995).

1.3.2 KSHV and other lymphoproliferative diseases

Besides KS, KSHV sequences have also been associated with other lymphoproliferative disorders and multiple myeloma. Viral sequences have been consistently found in primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Cesarman *et al.* 1995a, Cesarman *et al.* 1996a; Nador *et al.* 1996; Gaidano *et al.* 1996; Soulier *et al.* 1995). KSHV has also been

associated with multiple myeloma. These findings are rather controversial, as several groups were unable to confirm this association.

1.3.2.1 Primary effusion lymphoma

Primary effusion lymphoma (PEL), also called body cavity based lymphoma (BCBL), is a rare form of AIDS-associated B cell lymphoma and was initially recognised in HIV infected individuals (Nador *et al.* 1996; Knowles *et al.* 1989). It is seen predominantly in patients with advanced stages of immunosupression (Komanduri *et al.* 1996) but cases in HIV negative patients have been reported (Said *et al.* 1996; Nador *et al.* 1996).

The lymphoma is characterised by malignant effusion in pleural and abdominal cavities (Jaffe, 1996), usually without significant tumor mass or lymphadenopathy. Tumor cells are derived from a B cell lineage, as indicated by clonal immunoglobulin rearrangements and plasma differentiation markers. The majority of cells are co-infected with EBV and KSHV (Cesarman *et al.* 1995b; Gessain, 1997). However, some cases have been reported where only KSHV is present (Carbone *et al.* 1996; Arvanitakis *et al.* 1996). Compared to the endothelial and spindle cells in KS lesions, KSHV DNA sequences have been reported to be 50 times more abundant in PEL tumor cell lines (Cesarman *et al.* 1995b; Knowles *et al.* 1989). Analyses have shown that PEL cells are infected with multiple episomal copies of KSHV, 50 to 150 copies per cell have been detected, whereas KS cells may contain less than one copy per cell (Cesarman *et al.* 1995b). Unlike Burkitts lymphoma PEL cells lack c-myc rearrangement when

KSHV is present (Nador *et al.* 1996). Several B cell lines have been established from PEL patients and from the peripheral blood of PEL patients to study the pathogenesis of KSHV as well as to perform sero epidemiological studies. It is likely that these B cells may be similar to EBV transformed lymphoblastoid cell lines (LCL) and produce antibodies, although this has not yet been described. Some of these established B cell lines are positive for only KSHV (e.g. BCP-1, Boshoff *et al.* 1998) others are dually infected (e.g. HBL-6, Gaidano *et al.* 1996) The majority of these B cells are latently infected with KSHV, with approximately 2% of cells spontaneously entering the lytic replication cycle to produce infectious virions (Renne *et al.* 1996; Miller *et al.* 1997; Zhong *et al.* 1996). Several research groups have shown that the addition of phorbol ester or sodium butyrate to KSHV-infected PEL cell lines leads to the activation of viral gene expression (Renne *et al.* 1996; Moore *et al.* 1996a). As shown by Miller and colleagues in dually infected PEL cells (here the BC-1 cell line) the replication of each virus can be differentially induced by chemicals such as TPA (EBV) or n-butyrate (KSHV) (Miller *et al.* 1997). In co-infected cell lines the expression of EBV latent proteins is restricted to EBNA-1 and low levels of LMP-2 (Horenstein *et al.* 1997; Szekely *et al.* 1998). There are three stages of EBV latency, each type characterised by the expression of certain latent antigens. Burkitt's lymphoma is an example for latency type I, where EBNAI (essential for EBV replication) is expressed and lymphoma cells show c-myc translocation. In contrast, the transforming genes LMPI and EBNA2 are only expressed in type II and type III latency stages. Therefore the expression pattern seen in PEL cells corresponds to EBV latency

type I. It has been suggested that EBNA-1 and KSHV in combination might have the same transformation potential as EBNA-1 with c-myc translocation in Burkitt's lymphoma cells (Boshoff *et al.* 1998). Overall the interaction and specific roles of EBV and KSHV in PEL is poorly understood.

1.3.2.2 Multicentric Castelman Disease (MCD)

Multicentric Castelman disease, also called multicentric angiofollicular hyperplasia, is an atypical lymphoproliferative disorder described by Castelman in 1956 (Castleman *et al.* 1956). The disease is characterised by generalised lymphadenopathy and immunological abnormalities. Two histological forms can be distinguished; the hyaline-vascular variant and the plasma variant. Patients with MCD tend also to develop non-Hodgkin's lymphoma or KS. The factors involved in MCD development have not yet been established. However, high levels of IL-6 have been found and suggested to play an important role in disease development (Leger Ravet *et al.* 1991).

KSHV has been detected in 50% of the plasma variant when HIV-infected, but HIV-negative cases have been reported (Soulier *et al.* 1995). In particular PBMCs from MCD patients have been shown to harbour the virus (Soulier et al., 1995). Recently, large immunoblastic cells belonging to the B-cell lineage have been found to harbor the viral latent nuclear antigen (LANA) (Dupin *et al.* 1999).

1.3.2.3 Multiple Myeloma

The identification of KSHV sequences in bone marrow stroma cell cultures from patients with multiple myeloma has been controversial. Some groups have detected viral sequences by PCR, RT-PCR and in situ hybridisation methods (Rettig *et al.* 1997; Said *et al.* 1997b; Brousset *et al.* 1997; Agbalika *et al.* 1998). Furthermore, using serological assays, antibodies against lytic and latent KSHV antigens could be detected in multiple myeloma patients (Gao *et al.* 1998). However, others could not confirm these findings and further serological studies were unable to identify KSHV antibodies (Yi *et al.* 1998; Parravicini *et al.* 1997; Tisdale *et al.* 1998; Masood *et al.* 1997). Given these conflicts a link between KSHV and multiple myeloma remains to be established.

1.4 Epidemiology of KSHV

Polymerase chain reaction and serological studies have been used to define the epidemiology of the newly identified herpesvirus KSHV. However, the exact prevalence is still unclear, as different serological assays show high inter-assay variability with low-titre sera from non-KS patients. Current knowledge suggests that KSHV is not ubiquitous in Northern Europe, North America and some parts of Asia, but is relatively common in Southern Europe (Italy, Greece) and Africa (Calabro *et al.* 1998; Gao *et al.* 1996b; Kedes *et al.* 1996; Mayama *et al.* 1998; Simpson *et al.* 1996; Whitby *et al.* 1998).

1.4.1 Establishment of serological assays

Several serological assays detecting antibodies to KSHV lytic or latent antibodies have been developed. In particular the latent nuclear antigen LANA (Gao *et al.* 1996a), encoded by open reading frame 73 (Rainbow *et al.* 1997; Kellam *et al.* 1997) has been widely used to analyse KSHV sero-prevalence. Antibodies to LANA can be detected by western blot or by indirect immunofluorescence assays (IFA) using an noninduced PEL cell line (Gao *et al.* 1996b; Kedes *et al.* 1996). In IFA, KSHV positive serum gives a characteristic nuclear 'stippling' pattern due to the presence of anti-LANA antibodies (Kellam *et al.* 1997). Antibodies directed against LANA have been found in 80-85% of AIDS associated KS, and in more than 90% of classical KS, but only in 0-3% of US and UK blood donors (Gao *et al.* 1996b; Kedes *et al.* 1996; Simpson *et al.* 1996; Calabro *et al.* 1998; Lennette *et al.* 1996; Chandran *et al.* 1998). Other assays are based on cell extracts derived from chemically-induced B lymphoid cell lines, and mainly detect viral structural proteins. Their sensitivity and specificity is reported in US blood donors of between 0% (Smith *et al.* 1997) to 25% (Lennette *et al.* 1996). However, it has been demonstrated that serological cross reactivity with other viral proteins, for example the EBV structural proteins, can occur (Andre *et al.* 1997) making these assays potentially less reliable.

Enzyme linked immunabsorbent asays (EIA) have been established using virions purified from chemically-induced PEL cell lines (Whitman *et al.* 1997).

Several recombinant viral proteins have been generated and tested in serological assays. The viral open reading frame 65 encodes a capsid related protein which is able to detect antibodies in 75-80% of AIDS KS patients and in 85-90% of

patients with classical KS. Only 1-3% normal blood donors in the UK and 5% in the US tested positive with this assay (Simpson *et al.* 1996). Recently, a polypeptide encoded by KSHV ORF8.1 has been described as frequently recognized by KS patient sera on Western Blots with induced PEL cells. Antibodies to K8.1 could be detected in 80-90% of 16/20 KS patients, whereas control sera did not show any cross reactivity (Li *et al.* 1999). Combining all these different latent and lytic antigens may be an option for use in diagnostic assays. A study comparing the reactivity of different latent (LANA) and lytic (ORF65; K8.1) viral antigens in IFA and western blot analyses showed that a combination of both assays may be a more reliable way of detecting KSHV antibodies (Zhu *et al.* 1999). In addition several monoclonal antibodies have now been produced, including anti-ORF73, and anti-ORF8.1, these will be useful tools in future serological analyses as well as in studies of the pathogenesis of KSHV (Raab *et al.* 1998; Kellam *et al.* 1999; Chandran *et al.* 1998).

1.4.2 Sero-epidemiology in the general population and in HIV infected individuals in Western Countries

In the general population of North America, the UK and Japan KSHV sero prevalence in blood donors may be no greater than 1% (Gao *et al.* 1996a; Gao *et al.* 1996b). Depending on which antibody detection method has been applied, the prevalence rates among UK or US blood donors range from 0 to 3% (anti-LANA), 3-5% (anti-ORF65) and 0 to 25% when detecting undefined structural antigens (IFA) (Chandran *et al.* 1998; Lennette *et al.* 1996; Whitman *et al.* 1997). These

findings have been supported by PCR studies using PBMCs, samples from lymphoid tissue and semen samples from healthy semen donors. KSHV was not detected in PBMCs from UK, US and French healthy control individuals (Whitby *et al.* 1995; Moore *et al.* 1996c; Lefrere *et al.* 1996). In contrast the sero prevalence of KSHV is higher in some populations at risk for sexually transmitted diseases, with the highest prevalence in homosexual male groups (Gessain, 1997). Among HIV infected individuals antibodies against LANA can frequently be detected in homosexual patients (35%) as well as in 70-85% of HIV infected patients with KS (Kedes *et al.* 1996; Miller *et al.* 1996; Gao *et al.* 1996b; Lennette *et al.* 1996; Simpson *et al.* 1996). The KS incidence in HIV infected patients increases with age and is higher in male patients. Furthermore patients who reported homosexual intercourse with other males have significantly elevated risks for KS, both in Europe and the US (Kedes *et al.* 1996; Melbye *et al.* 1998). In contrast, groups with low risk for the development of KS (blood donors, HIV-positive women, intravenous drug users and haemophiliacs) show correspondingly low rates of sero-positivity (1-3%) (Kedes *et al.* 1997a; Simpson *et al.* 1996). Studies examining seroconversion and the presence of viral DNA in blood from HIV-infected gay men with no evidence of KS, have demonstrated that patients found to have KSHV are at a higher risk of developing KS in the future (Gao *et al.* 1996a; Moore *et al.* 1996c; Whitby *et al.* 1995). Overall, the high prevalence of KSHV among HIV infected homosexuals, together with the low rate of sero-positivity among other HIV risk groups, supports the hypothesis of a causative role of KSHV in KS.

1.4.3 Sero-epidemiology in Mediterranean Europe and Africa

High proportions of normal sera from those parts of the world associated with the classic and endemic forms of KS show a high detection rate of antibodies against LANA or ORF65. In Italy and Greece the virus is relatively abundant in the general population, reflecting the higher incidence of classic KS (5-20% in Italy and Greece) (Gao *et al.* 1996b; Kedes *et al.* 1996; Glenn *et al.* 1999). Also in semen samples from Italian semen donors the prevalence of KSHV is high (13-23%) (Blackbourn and Levy, 1997).

In Africa and the Middle East infection without disease is widespread. Up to 60% sero-positivity in Africa has been reported when using IFA on non-induced PEL cells. However, there are substantial regional variations, East and Central Africa, including Republic of the Congo, Uganda and Zambia have more cases of endemic KS than other parts of the continent. In Central Africa antibodies against LANA have been detected in more than 80% of the population (Kedes *et al.* 1996). In Uganda analysis of KS tumor lesions indicated a universal presence of KSHV DNA. Furthermore 85% of PBMCs of KS patients were positive when tested by PCR (Purvis *et al.* 1997). The transmission route of KSHV is different in Africa compared to Northern Europe and the US. While in Northern Europe and the US KSHV is more likely to be acquired through sexual contact after puberty (Melbye *et al.* 1998; Martin *et al.* 1998), in Africa, KSHV sero-positivity is common among children from East and South Africa (Mayama *et al.* 1998; Kasolo *et al.* 1997). Since the AIDS epidemic the incidence of KS has risen explosively. KS is now the most frequently reported tumor in several African countries (Wabinga *et al.* 1993), especially in Uganda, where childhood KS has

risen more than 40 fold (Ziegler and Katongole Mbidde, 1996). Studies suggest that vertical as well as horizontal transmission among children in Africa may play an important role in the route of infection (Angeloni *et al.* 1998). Usually the mother of an KSHV positive child will be positive as well. How the virus is transmitted to children is unknown, (possibly during child birth or breast feeding). It has been shown that saliva from PCR positive samples may contain infectious virus (Vieira *et al.* 1997). An interesting situation has been reported in The Gambia where KSHV infection is very common, but KS is rarely seen. Some cases have been described in AIDS patients infected with HIV-1 (Ariyoshi *et al.* 1998).

In summary, a consistent correlation between KSHV sero-prevalence and high-risk KS groups can be demonstrated (Gao *et al.* 1996a; Gao *et al.* 1996b; Kedes *et al.* 1996; Smith *et al.* 1997; Whitby *et al.* 1995). Also the detection of anti-LANA sero-positivity closely reflects the risk of developing KS in the future. KSHV does not seem to be ubiquitous in the general population, unlike most other herpesviral infections (Simpson *et al.* 1996; Neipel *et al.* 1998). Currently, KSHV seems to be confined to those populations at risk for developing KS.

1.5 KSHV morphology and genomic organisation

1.5.1 Morphology of KSHV

KSHV has morphological features typical of herpesviral capsids (see figure 1.1). Electron microscopy using induced primary effusion lymphoma cell lines revealed both intranuclear herpesvirus nucleocapsids and complete cytoplasmic virions.

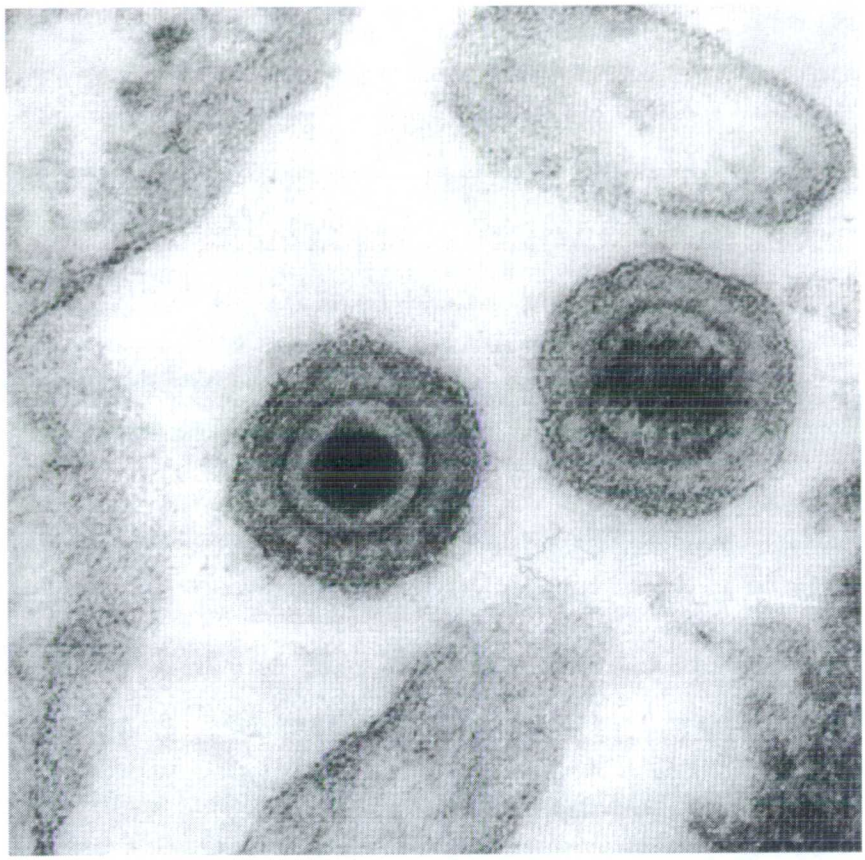

Figure 1.1: A KSHV particle from a primary effusion lymphoma cell line showing typical herpesvirus morphology, including core, envelope and tegument. Reprinted from Schulz, 1997.

Capsids are 110nm in diameter and surrounded by an envelope. Within the nucleus a nucleocapsid with an electro dense core can be detected (Said *et al.* 1997a; Renne *et al.* 1996). Similar morphological features typical of herpesviruses have also been observed in spindle cells from KS lesions (Orenstein *et al.* 1997; Said *et al.* 1997a).

1.5.2 Genomic organisation of KSHV

The genome of gammaherpesviruses exhibit a common structure. Large colinear genomic blocks, encoding typical herpesviral proteins (DNA polymerase, thymidylate kinase, capsid proteins), are interspersed by regions containing virus type-specific open reading frames (ORF) (Kieff, 1996). These unique open reading frames are predominantly involved in physiological processes and determine the pathogenesis of each virus. In figure 1.2 a schematic picture of the KSHV genome is shown. The complete sequence of KSHV has been determined from a BCBL derived cell line, BC-1 (Russo *et al.* 1996) as well as from a KS biopsy specimen (Neipel *et al.* 1997). The genome is a 190-kb double stranded DNA molecule consisting of a central, low-GC content, long unique region (LUR) of 140.5kb which is flanked each side by non coding tandemly repeated units. Each repeat is approximately 800bp in size and high in GC content (85%, HDNA). The genomic organisation of KSHV is very similar to other rhadinoviruses (Renne *et al.* 1996; Russo *et al.* 1996; Neipel *et al.* 1997b). Within the LUR 89 ORFs have been identified, 66 have sequence similarity to HVS open reading frames, the nomenclature for individual genes correspond to the HVS

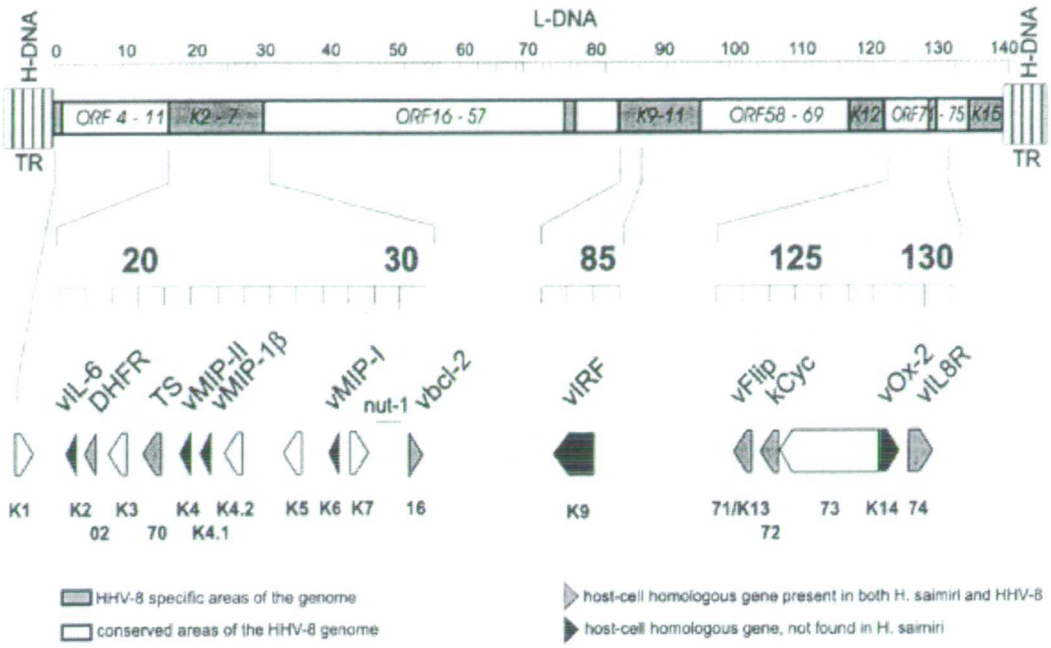

Figure 1.2: Structure of the KSHV genome. The central long unique region (LUR) is flanked by tandem repeats. The LUR contains at least 89 open reading frames, most of the genes have homologs in other herpesviruses. The conserved gene blocks (open boxes) are interspersed by non-conserved areas (gray boxes) contain genes with homology to other rhadinoviruses (v-cyclin, LANA, vGPCR) but also open reading frames unique to KSHV that have no similarity with other known viral genes. These 15 unique open reading frames have been designated with a K prefix (K1-K15). In the bottom of the picture genes with potential influence in KSHV pathogenesis are enlarged. Black arrows indicate homology with cellular genes; gray arrows symbolize homology with cellular genes and genes found in Herpesvirus saimiri, open arrows represent genes that are unique for KSHV. Reprinted from Neipel and Fleckenstein, 1999. For a more detailed genetic map see Neipel *et al.* 1997.

genome. KSHV shares the genomic block organization of other herpesviruses (Kieff, 1996). In KSHV the conserved genes are arranged in four or five blocks and include major structural proteins, DNA synthetic enzymes, glycoproteins and a viral proteinase and assembly protein. These genes are required for replication and assembly of new virus progeny (Unal *et al.* 1997; Russo *et al.* 1996). Between these blocks are non-conserved regions that show homology to other rhadinoviruses, e.g. the v-cyclin, LANA or vGPCR. In addition 15 open reading frames have been identified in KSHV that are unique and show no similarity with other known viral genes. These unique open reading frames have been designated with a K prefix (K1-K15).

A general feature observed in DNA viruses is the presence of open reading frames with significant homology to cell cycle regulatory and signalling proteins as well as proteins that can interfere with the immune system (Murphy, 1994). These genes are mainly unspliced and may have been captured from the host cell during viral evolution. In KSHV this 'piracy' of eukaryotic genes seems to be more extensive than is seen in any other herpesvirus (Moore *et al.* 1996a). The KSHV genome is equipped with several genes which encode proteins that allow the virus to escape cellular responses to viral infection.

On infection the host activates a number of anti-viral immune responses, including cell cycle arrest, apoptosis and mobilization of the immune system. KSHV has evolved proteins that have immunomodulatory functions such as Cytokines (vMIP-I; vMIP-II) (Moore *et al.* 1996a, Boshoff *et al.* 1997) and a homolog to Interleukin-6 (vIL-6) (Nicholas *et al.* 1997b; Moore *et al.* 1996a; Neipel *et al.*

1997a). Other proteins deregulate cell cycle mechanisms by interfering with receptor-mediated signal transduction pathways, including a homolog to the seven transmembrane cytokine receptor CXCR2 (vGPCR) (Cesarman *et al.* 1996b), a homolog to the interferon regulatory family (vIRF) (Gao *et al.* 1997; Moore *et al.* 1996a), and a D-type cyclin (v-cyc) (Cesarman *et al.* 1996b). A number of viral gene products also have transforming properties, e.g. open reading frame K1 (Lee *et al.* 1998b) and kaposin (K12) (Muralidhar *et al.* 1998). In addition ORF73, which encodes LANA, which has possible cellular growth-promoting activities (Kellam *et al.* 1997). To effectively suppress or delay apoptosis KSHV carries a bcl-2 homolog (v-bcl-2, ORF16) (Cheng *et al.* 1997; Sarid *et al.* 1997; Nicholas *et al.* 1997a) and a protein with anti-apoptotic function (vFLIP, ORFK13). Also viral homologs of cellular complement-binding proteins CD21/CR2 (ORF4) (Russo *et al.* 1996) and an NCAM-like adhesion protein (ORF14) (Russo *et al.* 1996) have been detected. The plethora of possible interactions between these proteins makes modeling the pathogenesis of KSHV problematical.

Apart from the proteins that may play a role in oncogenesis, several other proteins have been identified within the KSHV genome with importance for viral replication. Some examples include the processivity factor-9 (ORF59) that binds the viral DNA polymerase (ORF9) to allow synthesis of extended stretches of DNA without dissociation from the DNA template (Lin *et al.* 1998) and K8.1, a positional analog to

HVS ORF51, which encodes a glycoprotein expressed in lytic replication and present at the surface of infected cells and virion particles (Raab *et al.* 1998; Li *et al.* 1999).

1.5.2.1 Transcription pattern of viral genes

In PEL cells as well as KS biopsies only a small sub-population of cells actively replicates the virus, whereas the majority of cells retain KSHV as a latent episome (Renne *et al.* 1996; Russo *et al.* 1996). In these latently infected cells a subset of genes is expressed that appear to maintain the latent state of the virus (Miller *et al.* 1997; Sarid *et al.* 1998; Staskus *et al.* 1997). However, the lytic replication cycle can be induced in cell lines derived from primary effusion lymphoma by chemical stimuli such as TPA and n-butyrate. According to their transcription pattern three to four broad types of viral gene transcription have been identified: latent, immediate early, early and late (Sarid *et al.* 1998; Sun *et al.* 1999). LANA and the anti apoptotic protein vFLIP are expressed constitutively and their expression was not induced by the chemicals that induce lytic replication. During latency three transcripts have been detected. Latent transcript 1 (LT1) and latent transcript 2 (LT2) are differentially spliced homologous mRNAs and encode a cluster of genes suggested to have oncogenic properties (oncogenic cluster) (Sarid *et al.* 1998; Dittmer *et al.* 1998). The third latent transcript has not been characterized. LT1 encodes ORF73 (LANA-1), ORF72 (v-cyc) and ORFK13 (vFLIP), while LT2 only encodes ORF72 and ORFK13. Studies have revealed that the promoter

controlling the transcription of both transcripts is regulated in a cell-cycle dependent manner (Sarid *et al.* 1999).

In gamma herpesviruses some viral immediate early genes that can activate the transcription of lytic genes have been determined (Chevallier Greco *et al.* 1986; Nicholas *et al.* 1991; Whitehouse *et al.* 1998). So far two KSHV proteins that can regulate lytic expression have been identified. The ORF K8 encodes a protein belonging to the basic-leucine zipper (bZIP) family of transcription factors (Lin *et al.* 1999). This viral protein, designated K-bZIP, shows homology to the EBV BZLF1 (ZEBRA) gene. BZLF1 also contains a leucine zipper motif, which mediates binding to a group of target sequences (BZLF1-responsive elements) that are found in a number of EBV early promoters (Rooney *et al.* 1989). When overexpressed BZLF1 can reactivate latent EBV to enter the lytic cycle, indicating that it is a key factor in the reactivation and replication of EBV. In KSHV K-bZIP is a homodimerizing protein; its leucine zipper structure is generated through multiple splicing and transcription events and follows the kinetics of an early lytic gene (Lin *et al.* 1999). The gene product encoded by open reading frame 50 has also been identified as an immediate early gene that can activate early and late lytic gene expression from the latent viral genome (Lukac *et al.* 1998; Sun *et al.* 1998). This gene is a homolog of Rta, a transcriptional activator encoded by EBV (Sun *et al.* 1998). Among the gamma-herpesvirus family Rta proteins are well conserved (Albrecht *et al.* 1992; van Santen, 1993). These proteins are known to bind DNA specifically, but they may also interact with DNA indirectly via other proteins (Gutsch *et al.* 1994). KSHV/Rta has been shown to activate lytic cycle

gene expression in KSHV when PEL B cells were transfected with cloned KSHV/Rta and an increase of polyadenylated (PAN) RNA, mRNA for viral IL-6 was seen and an increase in the number of cells expressing capsid antigens could also be detected (Sun *et al.* 1998).

Early genes are transcribed without chemical stimulation in PEL cells but can be induced to a higher transcription level. A number of virally encoded cellular homologs (vIL-6, vMIP I, vMIP II), the antiapoptotic gene vBcl-2, and the putative cytokine receptor vGPCR have been shown to behave as early lytic genes (Sarid *et al.* 1998). The most abundant lytic cycle transcript was the polyadenylated RNA. PAN RNA (nut-1) does not appear to encode a protein (Zhong *et al.* 1996; Sun *et al.* 1996) but can associate with ribonuclear proteins (Zhong and Ganem, 1997). Late gene expression is indicated by e.g. the small viral capsid antigen (sVCA), the lytic immunogenic glycoprotein 8.1 and primarily by structural and replication genes that are transcribed to high levels only in response to chemical induction.

The pattern of mRNA expression in PEL cells is consistent with the transcription pattern observed in KS biopsies (Sun *et al.* 1999).

1.5.2.2 Variability of the genome

Overall the viral genome derived from B cells or spindle cells differ only 0.4% from each other (Neipel *et al.* 1997; Russo *et al.* 1996). Based on sequence comparison of ORF26 and ORF75 among different KSHV samples three distinct subtypes A, B and C have been described which differ by 1 to 1.5% at the

nucleotide level (Zong *et al.* 1997). However, the ORFK1 displays unusual high levels of genetic variability, resulting in four major genetic subtypes A, B, C and D. These clades differ by 15 to 30% at the amino acid level in this gene (Nicholas *et al.* 1998; Zong *et al.* 1997; Zong *et al.* 1999) and show distinctive ethnic and geographic association that are thought to have arisen during the migrationary divergence of modern humans in Paleolithic times (Zong *et al.* 1997). Most AIDS associated KS cases in the US are A1, A4 or C3 variants, whereas classical KS cases from the Middle east, Asia, Europe and the US are classified as C2 variants. Recently sequence analysis of six other loci across the KSHV genome, including three segments in the central conserved portions and three at the extreme right-hand confirmed this division into three or four clades (Poole *et al.* 1999).

1.6 Viral Equipment of genes for latency, transformation and tumorgenesis

As mentioned above KSHV encodes a number of proteins that bear homology to cellular genes/proteins involved in cell growth control, signal transduction pathways and other regulatory processes (Boshoff *et al.* 1997; Neipel *et al.* 1997b; Nicholas *et al.* 1997a). KSHV has evolved several strategies to overcome cellular defense mechanisms both to establish a latent infection and to produce virions. The identification of homologs to proto-oncogenes and cytokines provides evidence that KSHV is likely to be a transforming virus. In contrast to EBV, KSHV modifies the cell signaling and regulation pathways by introducing exogenous genes from its own genome, whereas EBV-encoded proteins induce the

expression of cellular proteins, leading to immortalization and transformation of cells (Arvanitakis *et al.* 1995; Birkenbach *et al.* 1993). There is a similarity between genes encoded by KSHV and those induced by EBV. The Epstein Bar nuclear antigen (EBNA) and the Latent membrane protein (LMP) induce human interleukin-6, cyclin D, a G protein coupled receptor, cellular bcl-2, and the complement-controlling protein CR-2. Both viruses seem to have developed different strategies to achieve the same goal of overcoming cell cycle arrest, apoptosis and activation of cellular immunity.

1.6.1 Latent nuclear antigen and latent membrane protein

Serological studies using sera from KSHV infected individuals showed that infection with KSHV is associated with the development of antibodies to a nuclear antigen. Typically, a punctuate nuclear immunofluorescence pattern can be observed with KSHV positive PEL cells when reacted against sera from KSHV positive patients (Lennette *et al.* 1996; Gao *et al.* 1996b; Kedes *et al.* 1997b; Kellam *et al.* 1997; Rainbow *et al.* 1997). Screening of cDNA libraries with KS positive sera identified the latent nuclear antigen (LANA or LNA-1) as the product of ORF73 (Kedes *et al.* 1997b; Kellam *et al.* 1997). LANA is transcribed as a single polycistronic message with two other growth promoting or anti apoptotic proteins, namely v-cyc and vFLIP (Kellam *et al.* 1997; Rainbow *et al.* 1997). Several studies demonstrated its presence in all tissues and cell types associated with KSHV infection (Dupin *et al.* 1999; Kellam *et al.* 1997). The protein is expressed as a large polypeptide with numerous repeated blocks of

acidic residues; the number of repeats can vary for different isolates (Rainbow *et al.* 1997). The immunogenic nature, physical structure, nuclear localization and latent expression suggest that the protein is analogous to EBV Epstein Barr nuclear antigens (EBNA). These nuclear antigens play a pivotal role in the maintenance and replication of the EBV episome and in cell transformation. At least 11 EBV genes are expressed in latent infection (EBNAs 1-6, LMP-1-3, and EBER 1 and 2), although only EBNA and LMP proteins are required for B cell transformation. EBNAs also transactivate a number of host and viral genes involved in the regulation of cellular physiology and growth (Farrell, 1995; Kieff, 1996). LANA seems to associate with different nuclear features compared to EBNA, as shown by confocal microscopy in coinfected cells (Szekely *et al.* 1998). LANA and KSHV DNA colocalize to mitotic chromosomes, indicating a possible role in the persistence of the KSHV episome (Ballestas *et al.* 1999).

The recently identified third latent transcript, LT3, is thought to code for a family of transmembrane proteins with similarities to EBV latent membrane protein-2A (LMP2A) and latent membrane protein-1 (LMP1) (Sarid *et al.* 1998; Glenn *et al.* 1999, Poole *et al.* 1999). Corresponding sequences have been localized to the extreme right region of the KSHV genome, between ORF75 and Terminal Repeats (TR), sequence analysis of several KSHV isolates has identified two highly diverged alternative allelic forms. This region, referred to as ORF-K15, encompasses complex spliced latent state genes and transcription was up regulated after phorbol ester treatment (Poole *et al.* 1999; Glenn *et al.* 1999). The encoded protein, named Latency associated membrane protein (LAMP) contains several

sequence motifs, including TNF receptor-associated factor (TRAF) binding sites, as well as up to 12 transmembrane domains and, presumably, a cytoplasmic domain (Glenn *et al.* 1999). These features are reminiscent of both EBV LMP2A and LMP1. Transfection studies indicate that the KSHV protein is localized on the cell surface and intracellular membrane. Furthermore, association between the LAMP cytoplasmic domain and TRAF-1, -2 and 3 have been observed.

In EBV, LMP1 exhibits a variety of activities in target cells, including transforming properties in rodent fibroblasts (Wang *et al.* 1985), induction of phenotypic changes in epithelial cells and inhibition of epithelial differentiation in vitro (Fahraeus *et al.* 1990). Moreover LMP1 is essential for B-cell immortalization (Kaye *et al.* 1996), induces up regulation of antiapoptotic proteins (such as bcl-2, Bcl-xL) (Henderson *et al.* 1991; Young *et al.* 1997) and increases cytokine production.

Downstream signaling is mediated via cytoplasmic domains which interact with molecules of the TRAF family (Devergne *et al.* 1996; Kaye *et al.* 1996; Mosialos *et al.* 1995; Eliopoulos *et al.* 1999) and the presence of a TRADD (TNF receptor-associated death domain) (Izumi and Kieff, 1997).

EBV LMP2A is known to be expressed in nasopharyngal carcinoma as well as latently in B cells of asymptomatic individuals. It has been suggested that LMP2A plays an important role in maintaining viral latency (Longnecker *et al.* 1993). As the KSHV encoded LAMP shows similarity to both LMP1 and LMP2A it may possess features of both EBV proteins.

1.6.2 Viral proteins targeting cellular proliferation or inducing transformation

1.6.2.1 ORF72 (v-cyc)

KSHV ORF72 is part of the "oncogenic cluster" in KSHV and encodes a D type cyclin homolog (v-cyc) with functions similar to cellular cell cycle control proteins (Cesarman *et al.* 1996b; Chang *et al.* 1996a). Some other rhadinoviruses (HVS, MHV68) and some oncogenic retroviruses also possess homologues to D-type cyclins (Nicholas *et al.* 1992; Virgin, *et al.* 1997).

Cyclins are involved in the regulation of cellular proliferation. Cellular cyclins associate with cellular kinases (cdk) forming an active complex. These complexes modulate progression of the cell into S-phase by phosphorylating cell cycle control proteins, including members of the retinoblastoma suppressor protein related family (pRB) and histones. Several cyclins have been described which associate with different kinases to promote the progression of cells through the division cycle. Each cyclin/cdk pair performs at a distinct stage of cell cycle progression, forming a complex regulated network (Morgan, 1997).

Cellular cyclin D is required for progression through the G1 cell cycle checkpoint, controlled by the tumor suppresser protein retinoblastoma associated protein (pRB). The cyclin/cdk complex phosphorylates pRB and ablates its ability to bind certain transcription factors, resulting in transcription activation and progression of the cell into the S phase (Whyte, 1995). Tumor viruses, like SV40, HPV16 and adenovirus have all evolved mechanisms to subvert the host cell cycle through direct binding to pRB and its subsequent inactivation (Jansen Durr, 1996). Others inactivate pRB indirectly, e.g. HTLV-1 or EBV. EBV encodes proteins (EBNA-LP, EBNA-2) that induce cyclin D2 overexpression (Sinclair *et al.* 1995;

Arvanitakis *et al.* 1995) which subsequently will inhibit pRB and p53 (Zacny *et al.* 1998). This inhibition of pRB has been attributed to the need for a virus to induce S-phase DNA synthesis in order to expand virion production. KSHV cyclin is expressed during latency and has been shown to be a functional cyclin capable of directing phosphorylation and inactivation of the retinoblastoma tumor suppressor protein as well as phosphorylation of histone H1 via cdk6 activation (Chang *et al.* 1996a; Godden Kent *et al.* 1997; Li *et al.* 1997; Moore *et al.* 1996a; Moore and Chang, 1998). Studies also demonstrated that v-cyc can overcome the G1-S block mediated by pRB in SAOS2 cells (Chang *et al.* 1996a). These cells have a homozygous deletion for pRB and upon co transfection of pRB and v-cyc continuous growth can be established. However, in contrast to cellular D type cyclin, viral cyclin in association with cdk6 can also phosphorylate histone H1 (Li *et al.* 1997; Godden Kent *et al.* 1997; Mann *et al.* 1999). Unlike cellular cyclins, v-cyc is resistant to certain cdk inhibitory proteins, e.g. the p27 (Kip) inhibitor is phosphorylated by v-cyc and in combination with cdk6 triggers p27 destabilization (Ellis *et al.* 1999; Swanton *et al.* 1997). This possibly allows the secondary transactivation of other p27-sensitive cyclin/cdks. Inactivation of p27 is a common feature in oncogenic virus infection, such as by adenovirus and human papillomavirus (Zerfass Thome *et al.* 1996; Mal *et al.* 1996).

Expression of viral cyclin has been detected in latently infected PEL cells as well as in KS spindle cells (Sturzl *et al.* 1999). In KS lesions v-cyc is present in 1% of spindle cells in early patches and in approximately 60% of the spindle cells in late

nodular lesions. Interestingly, v-cyc could also be detected in the peripheral blood of AIDS patients with KS (Davis *et al.* 1997).

So far v-cyclin has not yet been shown to have transformation abilities, but the viral cyclin may act in concert with other potential viral oncogenes and contribute to overall oncogenesis. Furthermore, deregulation of cdk activation has been implicated in the development of cancer (Bates and Peters, 1995).

1.6.2.2 ORFK1

Viral gene products exhibiting mitogenic and transforming properties have been identified in several gamma herpesviruses. These proteins are positioned at the same location in their respective genomes but do not share amino acid sequence similarity (Russo *et al.* 1996; Jung and Desrosiers, 1991). In the T cell specific oncogenic virus HVS two proteins have been associated with host cell transformation, the saimiri transformation associated protein (STP) and the tyrosine kinase interacting protein (TIP). STP is sufficient to immortalize primary T lymphocytes *in vitro* and induce lymphoma in marmosets (Duboise *et al.* 1998). TIP is associated with *ras* and the protein kinase C pathway (Lund *et al.* 1997). In KSHV no genes with sequence homology to the transforming genes TIP and STP have been identified. However, the open reading frame K1, the positional homolog to the HVS transforming protein STP, has been shown to induce morphological changes and alter growth of rodent fibroblasts. Furthermore, a recombinant HVS containing ORFK1 instead of STP, immortalized primary T lymphocytes and induced lymphoma in common marmosets (Lee *et al.* 1998b).

The structure of the K1 protein product is similar to lymphocyte receptors, and a functional immunoreceptor tyrosine-based activation motif has been identified. Furthermore, this cytoplasmic domain was found to induce cellular activation (Lee *et al.* 1998a).

1.6.2.3 ORFK12 (kaposin A)

A latency associated 0.7-kb transcript (T0.7) is expressed abundantly in all forms of KS, as well as in PEL cells (Zhong *et al.* 1996; Sturzl *et al.* 1997; Renne *et al.* 1996). It encodes two small open reading frames as well as ORF12, also known as kaposin (Russo *et al.* 1996; Neipel *et al.* 1997b). The T0.7 mRNA can be found in KS tissue of all stages (Staskus *et al.* 1997). In gene expression studies ORF12 was shown to be able to induce tumorgenic transformation (Muralidhar *et al.* 1998).

1.6.2.4 ORF74 (vGPCR, vIL8R)

The KSHV open reading frame 74 encodes a G protein coupled receptor (Cesarman *et al.* 1996b; Russo *et al.* 1996) with homology to the human chemokine receptors CXCR1 and CXCR2 (Murphy and Tiffany, 1991; Holmes *et al.* 1991).

Human chemokine receptors are a family of G protein-coupled receptors (GPCR), that are involved in the chemotaxis of leukocytes to other cells (Strader *et al.* 1995). The receptors couple extracellular stimuli to cell responses through guanine nucleotide binding proteins and can be subdivided according to their agonist-

specificity into two groups: C-X-C chemokine specific receptors binding, e.g. interleukin 8 (IL-8), and C-C chemokine receptors, which are specific for MIP-1α, MIP-1β, RANTES (Baggiolini *et al.* 1994). These two families of chemokines are discussed more fully below (section 1.6.5.2). However, the viral GPCR is able to interact with both CC and CXC chemokines, although it binds IL-8 with the highest affinity (Arvanitakis *et al.* 1997). *In vitro* studies have shown that the receptor exhibits an agonist-independent activity resulting in a constitutively active signaling molecule that can influence both proliferation and angiogenesis (Cesarman *et al.* 1996b). Overexpression of recombinant vGPCR induces tumorgenic transformation in rodent cells and vGPCR transformed NIH3T3 cells efficiently formed tumors in mice (Bais *et al.* 1998). KSHV ORF74 is primarily expressed during lytic replication (Kirshner *et al.* 1999) and plays an important role in neoangiogenesis and inflammatory cell infiltration (Bais *et al.* 1998). Signaling is mediated through the phosphoinositide-inositoltriphosphate-protein kinase C (PLC) pathway (Arvanitakis *et al.* 1997) and JNK/SAPK as well as the p38-MAPK protein kinase pathway. In infection it initiates a program of cellular gene expression that culminates in the synthesis and release of vascular endothelial growth factor (VEGF) (Bais *et al.* 1998). This growth factor and its receptor are regulators of angiogenesis. KS spindle cells express high levels of VEGF receptor and VEGF-3 (Jussila *et al.* 1998). Hence, VEGF has been regarded as a key factor in KS pathogenesis.

Although it has been demonstrated that the vGPCR is constitutively active, some chemokines and chemokine variants have been shown to modulate its activity

level. Inhibition of signaling has been observed for the viral encoded chemokine vMIP-II (CC chemokine), the cellular stromal cell derived factor-1-alpha (SDF-1α, a CXC chemokine that can block HIV-1 infection) and Interferon Gamma-inducible protein 10 (IP-10) (Geras Raaka *et al.* 1998b; Geras Raaka *et al.* 1998a; Gershengorn *et al.* 1998). In contrast, other chemokines have been shown to increase the level of signaling (Gershengorn *et al.* 1998). GPCRs homologs have also been identified in HVS (Ahuja and Murphy, 1993) and CMV, which encodes several GPCR homologs as lytic genes (Vieira *et al.* 1998; Welch *et al.* 1991).

In summary, the KSHV vGPCR, with its transformation potential and constitutive signaling ability, may be another viral oncogene. Furthermore, as a GPCR, HIV-1 gp120 may bind and signal through this receptor, which may contribute to the relatively aggressive disease course in HIV/KSHV co-infection.

1.6.3 Viral proteins and Interferon signaling

KSHV encodes four open reading frames that show homology to the transcription factors of the interferon regulatory factor (IRF) family (Li *et al.* 1998; Moore *et al.* 1996a; Burysek *et al.* 1999; Russo *et al.* 1996). So far two of these interferon regulatory factors (vIRFs) have been analysed, vIRF-1 and vIRF-2 (Gao *et al.* 1997; Li *et al.* 1998; Burysek *et al.* 1999). Interferons are a family of multifunctional cytokines with antiviral activities which interact with cell surface receptors, resulting in the synthesis of groups of cellular proteins (David, 1995). Stimulation of interferon synthesis is regulated at a transcriptional level and activation of interferon genes is mainly mediated by two families of

transcriptional factors, the NF-κB family and the interferon responsive factors, which include IRF-1, IRF-2, IFN-consensus sequence binding protein (ICSBP), and the IFN-alpha stimulated factor 3 gamma (Fujita *et al.* 1988; Harada *et al.* 1989). These transcriptional factors recognize a conserved cis-acting DNA element located within the promoter of this target genes (David, 1995). IRF-1 is a transcriptional activator, and IRF-2 its antagonistic repressor. Both have been identified as regulators of IFN-alpha and IFN-inducible genes (Harada *et al.* 1993). Studies have shown that the KSHV IRF-1 can function as a repressor on promoters which contain IFN-sensitive response elements. Expression of recombinant vIRF-1 (K9) dramatically repressed transcriptional activation induced by IFN-α, β and γ (Zimring *et al.* 1998). It has also been demonstrated that vIRF-1 can induce transformation in rodent fibroblasts, which when injected into mice form tumors (Gao *et al.* 1997; Li *et al.* 1998; Zimring *et al.* 1998). Furthermore, vIRF-1 plays an important role in regulating KSHV gene expression (Li *et al.* 1998). The second viral IRF (vIRF-2) has a different pattern of expression and properties from vIRF-1 (Burysek *et al.* 1999). The vIRF-2 interacts with cellular transcription factors and its expression can not be detected after chemical treatment of PEL cells (Li *et al.* 1998; Burysek *et al.* 1999). Both vIRFs show possible mechanisms which may interfere with IFN-mediated antiviral activity or deregulate the host immune system. Furthermore, vIRF-1 also has transforming properties and hence can be classified as another KSHV oncogene.

1.6.4 Viral proteins exhibiting anti-apoptotic properties

1.6.4.1 ORF16 (vbcl-2)

Programmed cell death (Apoptosis) is a common feature of mammalian cells and is important for embryonic development and the maintenance of homeostasis of multinuclear organisms (Vaux *et al.* 1994). This cellular self-destruction can also serve as a defense mechanism against viral infections to eliminate the infected cells, thereby slowing down viral spread. Viruses have evolved mechanisms to inhibit this host response to facilitate persistent infection or prolong the survival of lytically infected cells (Lewis *et al.* 1996; Crook *et al.* 1993). In contrast, others induce apoptosis and contribute to the resulting disease and mortality by destroying cells, e.g. apoptosis of $CD4^+$ cells and neurons in AIDS pathogenesis (Ameisen, 1995).

During some viral infections the tumor suppressor protein p53 is up regulated and activates apoptosis mediating proteins (the bcl-2 family) (Sato *et al.* 1994; Boyd *et al.* 1995). The bcl-2 protein family is characterised by the ability to modulate cell death, some promote cell death (Bax, Bak) whereas others inhibit apoptosis (bcl-2, bcl-xL) (Reed, 1996). Cellular bcl-2 has been implicated as a proto oncogene in the pathogenesis of human follicular lymphomas and other cancers (Cleary and Rosenberg, 1990; Cleary, 1991) via its anti apoptotic effect. Studies revealed that the bcl-2 protein interacts with other proteins with which it shares amino acid homology and overcomes bax-mediated apoptosis (Sato *et al.* 1994).

Homologs of bcl-2 have been identified in a number of γ-herpesviruses, including EBV (BHRF1) and HVS (ORF16). The bcl-2 homolog encoded by EBV is dispensable for the transformation of B lymphocytes but is able to block cell

differentiation in epithelial cells (Dawson *et al.* 1998). The ORF16 of KSHV has been shown to encode a bcl-2 homolog (Russo *et al.* 1996; Neipel *et al.* 1997b) and its expression during the lytic replication cycle has been demonstrated (Sarid *et al.* 1997). Its apoptosis suppressor activity has been proven using the Sindbis virus vector system (Cheng *et al.* 1997) and the viral protein is able to block apoptosis as efficiently as cellular Bcl-2 and Bcl-xl. However, KSHV bcl-2 does not homodimerize or heterodimerize with other bcl-2 family members, thereby escaping the regulatory effects of other cellular bcl-2 family proteins (Cheng *et al.* 1997).

1.6.4.2 ORFK13/73 (vFLIP)

It has been suggested that apoptosis can be mediated through the activation of the ICE-like family of cysteine proteases (caspases) (Henkart, 1996). Initially the tumor suppressor p53 may up regulate the expression of the so called "death" receptors (CD95, TNFR-1, TRAMP) along with their ligands (Nagata, 1997; Bodmer *et al.* 1997; Kitson *et al.* 1996). For example, CD95 (also called Fas or Apo-1) triggers a cell to apoptose, together with its ligand CD95L, in response to virus specific cytotoxic T lymphocytes (Thome *et al.* 1997). Death receptors belong to the TNF receptor superfamily and encode a cytoplasmic death domain (DD) which interacts with the death domain of adapter proteins (FADD, TRADD) (Nagata, 1997). FADD then associates via its death effector domain (DED) with the ICE-like protease FLICE, leading to the assembly of a receptor (CD95)-associated death inducing signalling complex (DISC) (Kischkel *et al.* 1995).

Finally DISC-associated FLICE initiates proteolytic activation of other ICE protease family members, which in turn leads to programmed cell death (Muzio *et al.* 1996; Medema *et al.* 1998).

The KSHV ORFK13/71 encodes an inhibitory protein that interferes with this pathway (Thome *et al.* 1997). Homologs have been identified within several gamma herpesviruses (EHV-2. BHV-4, HVS, KSHV). These FLICE inhibitory proteins (vFLIPs) contain two DED domains which interact with the FADD adapter protein, thereby inhibiting the recruitment and activation of the protease FLICE (Thome *et al.* 1997). Studies showed that cells expressing vFLIPs are resistant to death receptor mediated apoptosis (Bertin *et al.* 1997; Thome *et al.* 1997). Similarly, tumor cells such as melanomas and hepatomas do not respond to CD95L-mediated apoptosis (Hahne *et al.* 1996). Thus, vFLIPS may not only promote viral persistence but also contribute to the transformation capacity of KSHV and other gamma herpesviruses.

1.6.5 Viral cytokines and chemokines that support KS pathogenesis

As described earlier, the development of KS was suggested to be dependent on inflammatory cytokines and growth factors. Interestingly, KSHV encodes a number of cytokines that have been implicated in disease progression.

1.6.5.1 ORFK2 (vIL-6)

Before the discovery of KSHV the cytokine interleukin 6 (IL-6) was thought to play an important role in the development and progression of the human diseases with which KSHV has now been associated. AIDS-KS cell lines have been reported to secrete high amounts of IL-6 and to contain a large number of IL-6 receptors (Miles *et al.* 1990). Furthermore, IL-6 was described to support the proliferation of KS cells in vitro (Miles *et al.* 1990). Elevated levels of IL-6 have also been detected in MCD lesions and in KS patient sera (Ishiyama *et al.* 1994). Recently, IL-6 has been shown to be important as an autocrine growth factor for PEL cells (Asou *et al.* 1998).

Human IL-6 normally functions in the regulation of the immune system and other tissues. It activates acute phase genes and is involved in the stimulation of B cells (Ihle, 1995). IL-6 binds to gp130, a part of the IL-6 receptor, and signals through a variety of transcription factors, including STAT (Ihle, 1995). Human IL-6 has been implicated in a variety of B cell neoplasms. The KSHV genome encodes an IL-6 homolog (ORFK2) which is expressed in approximately 50% of latently infected BCP-1 cells and in primary ascitic PEL, suggesting constitutive expression in latently infected PEL cells (Asou *et al.* 1998; Moore *et al.* 1996). However, vIL6 is only expressed by a minority of cells within the KS lesion, but can be found in hematopoietic cells and lymph nodes of KS patients (Moore *et al.* 1996a; Parravinci *et al.* 1997). KSHV encoded IL-6 is functional in a number of Il-6 dependent bioassays, as it promotes survival of IL-6 dependent cell lines (Burger *et al.* 1998; Moore *et al.* 1996a; Nicholas *et al.* 1997a). Although there are functional similarities to human IL-6, there are receptor binding differences (Wan

et al. 1999). Biologically, vIL-6 triggers the Janus Tyrosine Kinases/ signal transducers and activators of transcription (JAK/STAT) pathway via interactions with the IL-6 receptor as demonstrated in HepG2 cells. However, the gp130 signal transduction subunit alone is sufficient for viral IL-6 signaling (Molden *et al.* 1997). The low expression rate of vIL-6 in KS lesions suggests that vIL-6 plays a more indirect role in KSHV pathogenesis; perhaps in a paracrine manner.

1.6.5.2 ORFK4, K4.1, K5 (vMIP-I and vMIP-II)

Chemokines have an important role in leukocyte migration and activation as well as hematopoeisis (Mackay, 1996). Furthermore, several chemokine receptors have been identified as co factors for HIV entry into $CD4^+$ cells and some CC and CXC chemokines can block HIV entry into T cells, including the MIP/RANTES family (Cocchi *et al.* 1995; Dragic *et al.* 1996). As described before (section 1.6.2.4) there are two groups of pro-inflammatory chemokines; the CC chemokines (MIP-1α, MIP-1β, MIP-2, MIP-3 and RANTES), and the CXC chemokines such as IL-8 and SDF-1 (Baggiolini *et al.* 1994). The activity of these small soluble proteins is mediated through GPCRs. MIP-1 proteins are chemoattractants for monocytes, 1β acting preferentially on CD4+ T lymphocytes and 1α also on B lymphocytes and cytotoxic T cells (Schall *et al.* 1993). KSHV encodes three proteins with homology to cellular CC chemokines: vMIP-I; vMIP-II and vMIP-III (Moore *et al.* 1996a; Russo *et al.* 1996; Neipel *et al.* 1997b; Nicholas *et al.* 1997a). These virally encoded chemokines bind to both CC and CXC chemokine receptors, unlike cellular chemokines (Boshoff *et al.* 1997). Both vMIP-I (ORFK6) and

vMIP-II (ORFK4) show sequence similarity to human chemokine MIP-1α (Moore *et al.* 1996a), whereas vMIP-III (ORFK4.1) is less homologous. Studies demonstrated HIV-1 inhibitory activity for vMIP-1 and vMIP-2; vMIP-1 directly via interaction with the CCR5 chemokine receptor (which binds human MIP/RANTES) and vMIP-2 by association with CCR3 (which binds eotaxin) . The latter one has also been reported to bind to other chemokine receptors and may or may not be associated with chemoattraction of e.g. eosinophils (Moore *et al.* 1996a; Kledal *et al.* 1997; Boshoff *et al.* 1997). Furthermore, vMIP-II has been shown to bind the KSHV G protein coupled receptor and inhibits signaling (Geras Raaka *et al.* 1998a). Less is known about vMIP-I, this viral chemokine binds specific to the CCR8 chemokine receptor and induces signaling. These receptors are predominantly expressed on T helper 2 lymphocytes, indicating that vMIP-I may influence the host immune response (Endres *et al.* 1999; Dairaghi *et al.* 1999). Interestingly, vMIP-II also binds CCR8 as an antagonist without signal induction (Dairaghi *et al.* 1999). In a chorioallantoic assay both viral chemokines showed angiogenic characteristics and may therefore play a direct role in KS pathogenesis (Boshoff *et al.* 1997). Transcripts have been found in KS spindle cells but only in a few lytically infected cells (Boshoff *et al.* 1997). In latently infected PEL cells basal expression can be detected and can be further induced by TPA (Moore *et al.* 1996a).

The third viral chemokine, encoded by ORF4.1, is related to MIP-1β and is a macrophage chemoattractant (Neipel *et al.* 1997b; Nicholas *et al.* 1997a). In summary, the viral encoded chemokines may induce angiogenesis but may also be

responsible for inflammatory infiltrates. Also the influence of these vMIPs in the inhibition of HIV entry may influence disease progression in patients infected with both HIV and KSHV. The viral and human chemokines may compete with HIV-1 gp120 for the binding to T cells. The survival of AIDS-KS patients has been reported to be longer compared to AIDS patients without KS (Moore et al., 1996a; Sarid et al., 1999).

1.7 KSHV and Pathogenesis, a Hypothesis

KSHV is present in the vast majority of spindle cells in both early and late KS lesions. Most cells are latently infected but a few spontaneously enter lytic replication and produce viral particles. In EBV infection a similar scenario can be observed. Here, latently infected cells express viral genes that are responsible for genetic alterations leading to tumor growth. Perhaps this situation can be transferred to KSHV pathogenesis.

Several genes within the KSHV genome encode proteins with sequence and more importantly, functional homology to human oncogenes that have been implicated in several forms of human cancers. Furthermore, viral proteins with transforming potential have been identified. In Table 1.2 an overview of the KSHV genes possibly involved in viral-associated diseases is given. KSHV targets the checkpoint of progression through the G1 cell cycle encoding a functional homolog to the cell cycle control protein v-cyc. This feature, of triggering cell proliferation of the host cell despite a viral infection, has evolved in other DNA viruses, such as HPV, adenoviruses and the polyomaviruses. These viruses all

Table 1.2: KSHV encoded genes which exhibit possible oncogenic properties

KSHV ORF/name	Homologs	Functional studies	Possible role in KS pathogenesis	Latent/ lytic
K1	Positional homolog to HVS transforming protein (STP)	Lymphocyte immortalization, lymphoma induction	Transformation	Lytic
K2/vIL-6	Human IL-6 (hIL-6)	Proliferation of B cells	Paracrine growth stimulation of spindle cells	lytic
K4, K4.1, K5/vMIP	Human macrophage inflammatory protein (MIP)	Binding to both CC and CXC receptors Angiogenisis Attraction of eosinophils	Angiogenesis	lytic
K9/vIRF	Human interferon responsive factor (IRF)	Repressor on interferon activity Transformation of 3T3NIH cells; tumor in nude mice	Interferes with interferon mediated anti-viral activity; transformation	lytic
K12/ KaposinA		Transformation of Rat-3 cells	Transformation	latent
ORF16/ v-bcl-2	bcl-2	Blocks apoptosis in Sindbis virus vector system	viral persistence	lytic
ORF71/ vFLIP	Homolog in HVS	Anti apoptotic, resistant to death receptor mediated apoptosis	viral persistence, contribute to transformation ?	latent
ORF72/ v-cyc	D type cyclin	Functional cyclin, activates cdk6, assosiates with other molecules, resistant to CDK-inhibitors, destabilizes p27	Deregulation of cell cycle progression	latent
ORF73/ LANA	EBV EBNA	Immunogenic Colocalizes with KSHV DNA	persistence of KSHV episome	latent

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K9/vIRF	Human interferon responsive factor (IRF)	Repressor on interferon activity Transformation of 3T3NIH cells; tumor in nude mice	Interferes with interferon mediated anti-viral activity; transformation	lytic
K12/ KaposinA		Transformation of Rat-3 cells	Transformation	latent
ORF16/ v-bcl-2	bcl-2	Blocks apoptosis in Sindbis virus vector system	viral persistence	lytic
ORF71/ vFLIP	Homolog in HVS	Anti apoptotic, resistant to death receptor mediated apoptosis	viral persistence, contribute to transformation ?	latent
ORF72/ v-cyc	D type cyclin	Functional cyclin, activates cdk6, assosiates with other molecules, resistant to CDK-inhibitors, destabilizes p27	Deregulation of cell cycle progression	latent
ORF73/ LANA	EBV EBNA	Immunogenic Colocalizes with KSHV DNA	persistence of KSHV episome	latent

bind to the tumor suppresser proteins pRB or p53 and overcome growth arrest, EBV also which induces cellular G1 cyclins. The KSHV cyclin is transcribed as a latent protein and probably prevents replication to allow virus progeny production. Most spindle cells in KS lesions are infected latently, suggesting a direct role for KSHV in pathogenesis via latent growth-promoting viral proteins. Furthermore, other proteins encoded by KSHV also maintain or restore proliferation competence. The viral GPCR is able to transform NIH3T3 cells and induce production of a potent angiogenic factor, the vascular endothelial growth factor (Arvanitakis *et al.* 1997; Bais *et al.* 1998) and viral chemokines have been identified that also induce angiogenesis. Other growth stimulating factors, like vIL-6 may recruit uninfected cells as target for *de novo* infection. Cellular IL-6, known as a B cell growth factor, has been shown to stimulate KS cells *in vitro* and increased levels of IL-6 have been detected in Castleman's disease. In the pathogenesis of KS, the autocrine and paracrine growth effects of cytokines, growth factors and their receptors have been suggested to play an important role. Therefore, KSHV, with its own chemokines and the chemokine receptor, may satisfy this growth factor theory in itself and contribute to the development of KS. KSHV may also enhance the growth of latently infected cells by creating a favorable growth milieu resulting from the concerted activity of viral encoded proteins secreted by adjacent cells undergoing lytic replication.

However, some of these potential oncogenes, such as vGPCR, vIRF and the transforming gene K1, are unlikely to be transcribed during latency. Three latency associated transcripts have been identified that encode LANA, kaposin (possibly

transforming), v-cyclin and the anti-apoptotic protein vFLIP. The latency associated antigen LANA is thought to maintain the viral episome, vFLIP has been demonstrated to prevent programmed cell death and could be active both in maintaining proliferation and in blocking antiviral cytotoxic T cell-induced apoptosis.

In summary, epidemiology, the consistent presence of KSHV DNA in tumor tissue, the number of viral oncogenes and chemokines so far identified, and the fact that KSHV sero conversion can predict tumor development, all favor this virus as at least a major co-factor in tumor pathogenesis. Recently, a KSHV gene with directly transforming properties, ORFK1, a homolog to EBV EBNA1 and LMP2A, has been identified, which further support the hypothesis that KSHV is an oncogenic virus.

In contrast, PEL cells are commonly co-infected with KSHV and EBV, representing the first example of a consistent dual herpesviral infection. The latent transcription pattern of EBV in PEL is restricted, with mainly EBNA1 and no, or very low, levels of major EBV growth transforming factors (Horenstein *et al.* 1997). Also the in vitro transformation of PBMCs with KSHV indicated the importance of the presence of EBV for transformation (Kliche *et al.* 1998). How EBV and KSHV interact is hardly studied; but the presence of both viruses in almost all cell lines derived from PEL indicates a possible link in tumorgenesis.

1.8 Propagation of KSHV *in vitro*

1.8.1 B cell lines established from PEL patients

Attempts to cultivate KSHV *in vitro* have proven to be difficult. Cells derived from KS lesions tend to lose the viral genome upon passage (Aluigi *et al.* 1996; Ambroziak *et al.* 1995). Several B cell lines derived from PEL have been established, of which most are dually infected with KSHV and EBV. In PEL the viral genome is maintained in the nucleus as a circular episome. Viral replication can be subdivided into latent and lytic replication, during latency only a small proportion of cells spontaneously undergo lytic replication and produce infectious virions. The EBV expression pattern is restricted, with abundant levels of EBNA-1 and low LMP-1 and LMP-2A levels (Horenstein *et al.* 1997). It has been suggested that this combination of KSHV and EBNA-1 may have a similar transformation potential to c-myc translocation and EBNA-1, as seen in Burkitts lymphoma (Boshoff *et al.* 1998). Lytic gene expression of KSHV and EBV can be individually enhanced by treatment with phorbol ester (EBV) or butyrate (KSHV) (Miller *et al.* 1996).

Some B cell lines are only infected with KSHV, such as BCBL-1 and BCP-1, which were established from the peripheral blood of a PEL patient (Renne *et al.* 1996; Boshoff *et al.* 1998). Both cells also exhibit similar growth properties in SCID mice and immunoblastic lymphomas could be observed (Boshoff *et al.* 1998; Picchio *et al.* 1997). However, since these B cell lines are already latently infected in culture, studies on de novo viral entry and pathogenesis are difficult.

1.8.2 *De novo* infection

KSHV has been passaged in primary B cells but few other primary cell lines, some showing a productive infection. In general the genome is lost with subsequent passage. Limited replication has been reported for the human embryonic kidney cell line (293) with and without cytopathic effects (Foreman *et al.* 1997; Vieira *et al.* 1997; Renne *et al.* 1998). Others have tested a diversity of cell lines and demonstrated infectivity for BCBL-1 derived KSHV. Some of these cell lines, including cells of fibroblastic, epithelial, endothelial or lymphoid origin were susceptible to the virus but no CPE was produced and the level of infection was low (Renne *et al.* 1998). More successful viral transmission have been reported for primary human endothelial cells, including both primary and immortalized dermal microvascular endothelial cells (DMVEC). A distinct biology of KSHV derived from KS lesions and from PEL cells have been described in vitro culture systems (Friborg, Jr. *et al.* 1998). Using the EBV negative Burkitt's lymphoma cell line, Loukes, transmission of KSHV from KS lesions could be established and further induced with TPA to produce progeny virions. Virus obtained from this infected cell line (named BKS-1) was able to infect 293 cells and a CPE was detected, as was apoptosis. In contrast, virus derived from TPA-induced BCBL-1 cells did not induce cell death in 293 cells and viral DNA sequences were lost in culture (Friborg, Jr. *et al.* 1998).

Human primary endothelial cells, in particular bone marrow microvascular endothelial cells (BMVEC) and primary human umbilical vein endothelial cells (HUVEC), were successfully infected with purified KSHV and supported lytic replication. Initially some cells showed a CPE and died, whereas others could be

maintained in culture. In the same experiments KSHV induced long term proliferation compared to uninfected control cultures, where cells suffered senescence. Using IFA KSHV nuclear antigens were detected in 1-5% of the total cell population, which led the authors to conclude a paracrine mechanism is responsible for the survival of uninfected cells (Flore *et al.* 1998).

Other researchers stably infected primary dermal microvascular endothelial cells (DMVEC) by co culture with KS-1 cells (Panyutich *et al.* 1998). Recently, a robust long term infection of HPV (E6 and E7) immortalized DMVECs has been reported (Moses *et al.* 1999). KSHV infection was monitored by PCR and lytic replication detected by RT-PCR as well as IFA. The majority of cells could be infected and maintained the viral genome indefinitely. While most of the cells remained latent, a small number supported viral replication. Chemical treatment also induced lytic gene expression. Interestingly, infected cells undergo phenotypic changes including spindle cell morphology and characteristics of a transformed phenotype, precisely the features that resemble KS lesions (Moses *et al.* 1999).

B lymphocytes can also be persistently infected with KSHV under certain conditions (Mesri *et al.* 1996; Kliche *et al.* 1998). Studies showed that the presence of EBV is necessary to immortalize PBMCs. Only PBMCs from EBV positive individuals could be immortalized by supernatant derived from BCBL-1 cells (Kliche *et al.* 1998). These immortalized cells possessed the phenotype of activated B cells, similar to EBV positive lymphoblastoid cell lines (LCL). The LCLs were persistently positive for both viruses, KSHV replication could be

induced and KSHV could be serially passaged. Table 1.3 gives an overview of currently established cell lines which supported a KSHV infection *de novo*.

Table 1.3: Overview of current culture systems to establish *de novo* KSHV infections

Cell line	origin of virus	serial propagation	support of viral replication	reference
HEK 293	KS lesions, BCBL-1, BKS-1, saliva from KS patient	partly, abortive infection	yes, some CPE detected	Renne *et al.* 1998 Vieira *et al.* 1997 Friborg, Jr. *et al.* 1998 Foreman *et al.* 1997
Loukes	KS lesions	yes	yes	Friborg, Jr. *et al.* 1998
Human endothelial cells (BMEC, HUVEC)	BC-3	yes (12-14 months)	yes, transformation and CPE	Flore *et al.* 1998
Primary DMVEC	KS cells	at least 16 passages (app. 80days)	limited productive infection	Panyutich *et al.* 1998
Immortalized DMVECs	BCBL-1	< 1 year	yes, transformation and CPE	Moses *et al.* 1999
PBMC	BCBL-1, BC-1	< 9 months	yes, immortalization in the presence of EBV	Kliche *et al.* 1998

1.9 Herpesviral glycoprotein H and L

The envelopes of herpesviruses are relatively complex structures with large numbers of viral glycoproteins. Some play a role in viral attachment to the host cells, others mediate penetration into the host cell and some are involved in viral egress. Many viral glycoproteins are expressed on the surface of infected cells and on the virion and they constitute major antigenic determinants for the cellular and humoral immune response of the host (Roizman, 1993; Spear, 1993).

Unsurprisingly, the entry process is complex and not fully understand. For the alphaherpesvirus subfamily a number of entry mediators have been identified that may also play a role in cell-to-cell fusion. The receptor(s) mediating EBV entry into B lymphocytes have been determined, but productive infection of epithelial cells with EBV in vitro remains a mystery. Each member of the herpesvirus family encodes a diverse number of glycoproteins that enables them to establish its own niche. For the herpes simplex virus 11 unique glycoproteins have been described, HCMV may express even more (Spear, 1993; Spaete *et al.* 1994). However, some glycoproteins seem to be highly conserved among all herpesviruses. Glycoprotein H and glycoprotein L are among the few viral glycoproteins which have functional homologs in all three classes of herpesviruses. Studies have demonstrated that these proteins form a complex with one another and are essential for virion infectivity as well as virus-induced cell fusion in vitro and in animals (Babic *et al.* 1996; Forrester Forrester *et al.* 1992; Farrell *et al.* 1994; Fuller *et al.* 1989; Haddad and Hutt Fletcher, 1989; Miller and Hutt Fletcher, 1988; Peeters *et al.* 1992; Liu *et al.* 1993b).

1.9.1 Viral entry and cell to cell fusion

Herpesviral entry into cells is best studied in the alphaherpesvirus subfamily, in particular for herpes simplex virus 1 (HSV-1). Viral entry into the host can be divided into attachment of the virus to the cell surface and subsequent fusion of the viral envelope with the plasma membrane (penetration). Both events are mediated by several viral glycoproteins (Spear, 1993; WuDunn and Spear, 1989). Due to the fact that so many glycoproteins are involved in attachment to the host cell it is unlikely that only a single receptor will be responsible. These proteins presumably act in concert and may use a number of different receptors (Handler *et al.* 1996; Johnson *et al.* 1990; Johnson and Ligas, 1988; Lee and Fuller, 1993). The initial interaction of HSV with the cell membrane is mediated by glycoprotein C and/or B binding to heperan sulfate proteoglycans on the cell surface in a relatively low affinity interaction (Shieh *et al.* 1992; Herold *et al.* 1994). Subsequently, glycoprotein D interacts with cellular receptor(s) and, together with glycoprotein B and the gH/gL complex, triggers pH-independent fusion of the viral envelope with the host cell plasma membrane (Whitbeck *et al.* 1997; Cai *et al.* 1988; Johnson *et al.* 1990; Lee and Fuller, 1993; Spear, 1993). A number of cellular proteins have been identified as HSV-specific surface receptors based on this interactions with glycoprotein D. The herpesvirus entry mediator, HVEM/HveA can mediate entry of HSV into a subset of human cell types, including CHO cells and T lymphocytes and its interaction with gD has been demonstrated (Montgomery *et al.* 1996; Whitbeck *et al.* 1997; Nicola *et al.* 1997, Roller and Rauch, 1998).

HveA is a member of the TNF/NGF receptor family that is associated with gene regulation events and anti-apoptotic signaling. Its expression pattern suggests a role in lymphoid development and immune responses (Gravestein and Borst, 1998). Recently, other surface proteins allowing HSV entry into CHO cells, independent of HveA have been detected (Geraghty *et al.* 1998). Originally identified as poliovirus receptor related proteins, PRR1 and PRR2 have now been renamed HveC and HveB (Lopez *et al.* 1995; Eberle *et al.* 1995; Geraghty *et al.* 1998; Warner *et al.* 1998). Both molecules belong to the immunoglobulin superfamily and HveC has been demonstrated to mediate entry of all alphaherpesviruses (Krummenacher *et al.* 1998). Herpesviruses also infect cells by cell-to-cell fusion, whereby an infected cell membrane fuses with that of its non-infected neighbor. The mechanism by which herpesviruses induce cell fusion is not clear (Spear, 1993). However, there is evidence that cell to cell fusion is distinct from the membrane fusion event during viral entry (Ejercito *et al.* 1968). For example, certain cell lines are highly susceptible to infection but resistant to HSV-induced cell fusion (Bzik and Person, 1981; Spear, 1993). Some viral glycoproteins are important for fusion but dispensable for entry, e.g. gE, gI and gM (Davis Poynter *et al.* 1994). *In vitro* studies using viral mutants revealed that glycoproteins B, D and the gH-gL complex are essential for fusion of the viral envelope with the plasma membrane (Cai *et al.* 1988; Fuller and Lee, 1992); Turner *et al.* 1998). Also cell surface heparan sulphates are involved in HSV mediated cell to cell fusion (Shieh and Spear, 1994). Furthermore, cells resistant to virus induced cell fusion became susceptible when they expressed the herpesviral entry mediator HveA (Terry Allison *et al.* 1998).

In EBV, infection studies of viral entry are based on B lymphocyte cell culture systems. Although epithelial cells are fully permissive for lytic infection *in vivo*, *in vitro* EBV infections are inefficient. Several attempts to culture EBV have been made with cell lines expressing the EBV receptor CD21, but infections were abortive (Kieff, 1996; Sixbey *et al.* 1983; Li *et al.* 1992).

To enter B lymphocytes the EBV encoded glycoprotein gp340/220 binds to CD21 on the cell surface, this interaction may lead to the activation of signal transduction pathways (Kieff, 1996). The CD21 receptor is a member of the immunoglobulin superfamily and naturally interacts with the C3d component of the complement complex (Nemerow *et al.* 1990; Tanner *et al.* 1987). However, penetration of the virus into B lymphocytes may require additional proteins, for example CD21 accessory proteins or other cellular proteins. EBV infection of epithelial cells transfected with CD21 is inefficient indicating that other proteins may be required for effective EBV infection (Kieff, 1996).

Following attachment EBV internalizes into cytoplasmic vesicles, subsequently the viral membrane fuses with the vesicle membrane and the nucleocapsid is released into the cytoplasm. In contrast, in Burkitt lymphoma cells the viral nucleocapsid is released directly into the plasma membrane instead of being initially internalized (Kieff, 1996). As described for alphaherpesviruses, gH and gL functional homologs have been identified for EBV and are directly implicated in the fusion processes (Miller and Hutt Fletcher, 1988; Yaswen *et al.* 1993). In EBV a third protein has been detected that associates with gH and gL and is important for B lymphocyte infection (Li *et al.* 1995; Spriggs *et al.* 1996). An additional component of the gH gL complex

has also been described for VZV and HCMV (Huber and Compton, 1997; Li *et al.* 1997).

1.9.2 Glycoprotein H

Glycoprotein H is highly conserved among human and animal herpesviruses. Homologs have been identified in representatives of all subgroups of herpesviruses (Keller *et al.* 1987; Heineman *et al.* 1988; Cranage *et al.* 1988; Gompels *et al.* 1988; Josephs *et al.* 1991; Nicolson *et al.* 1990; McGeoch and Davison, 1986; Pachl *et al.* 1989; Klupp and Mettenleiter, 1991; Scott *et al.* 1993; Pepper *et al.* 1996). Primary sequences are poorly conserved, with only a limited conservation being detected in the C terminus (Gompels *et al.* 1988). Identified gH proteins range in size from 706 to 743 amino acid residues for the beta- and gammaherpesviruses, to 838 to 841 for the alphaherpesviruses. Overall, members of the gH family exhibit a conserved protein structure. The hydrophobic N terminal region comprises an 'ER' signal sequence for translation on membrane bound ribosomes, second hydrophobic region, adjacent to the C terminus, may function as a membrane anchor. Greatest sequence similarity is observed in the C-terminal halves of the protein including residues surrounding four conserved cysteines, a conserved N-linked glycosylation site (NGTV) and a short cytoplasmic domain (Gompels and Minson, 1986; Klupp and Mettenleiter, 1991; McGeoch and Davison, 1986). Functionally, gH is an important factor in cell penetration and cell-to-cell fusion. Monoclonal antibodies specific for gH allow attachment of the virus but prevent entry; deletion mutants lacking gH have also been shown to have this effect (Forrester *et al.* 1992; Fuller and Lee, 1992; Keay and

Baldwin, 1991). Recombinant gH is incompletely processed and accumulates in the cytoplasm, in particular the peri nuclear membrane, and is most likely retained in the endoplasmatic reticulum or *cis* Golgi (Gompels and Minson, 1989; Cranage *et al.* 1988; Heineman *et al.* 1988; Forrester *et al.* 1991; Foa Tomasi *et al.* 1991; Pulford *et al.* 1994; Roberts *et al.* 1991). Furthermore, some immunologically important epitopes are not presented by the recombinant protein. When injected into mice, these recombinant proteins only elucidate a weak immune response. Animals challenged with virus showed no or little virus neutralisation (Roberts *et al.* 1991; Ghiasi *et al.* 1992).

Within the viral life cycle glycoprotein H is transcribed late in lytic infection (McGeoch and Davison, 1986) and appears to be a relatively minor component of the infected cell surface and virion envelope (Buckmaster *et al.* 1984). Unsurprisingly, as gH exhibits targets for virus-neutralising antibodies and intracellular over expression would be disadvantageous for the virus (Miller and Hutt Fletcher, 1988; Montalvo and Grose, 1986). Reports suggest that gH expression can be regulated by environmental conditions, but the exact mechanisms have not been described. Similarly, interactions between glycoprotein H and the host cell membrane could not be established and the molecular mechanism of how gH contributes to viral fusion remains unknown. From phylogenetic analysis of glycoprotein H it is known that the NGTV motif has been conserved over million of years (McGeoch *et al.* 1995). This may indicate an important function within the viral life cycle. However, site directed mutagenesis of the NGTV motif demonstrated no influence on HSV-1 mediated fusion events (Galdiero *et al.* 1997). In contrast, the C terminal tail of gH, which projects either into

the cytoplasm or towards the virus capsid, has been shown to play an important role in cell-to-cell spread (Browne *et al.* 1996; Wilson *et al.* 1994; Anderson and Gompels, 1999). HSV-1 recombinant viruses containing mutations within the serine-valine-proline motif (SVP) of the cytoplasmic tail entered Vero cells less efficiently and were unable to induce cell to cell fusion (Browne *et al.* 1996). The domains involved in entry processes remain to be characterised in detail. Within the external domain a hydrophobic stretch of amino acids, preceding the transmembrane domain has been associated with viral entry and polykaryon production (Galdiero *et al.* 1997; Liu *et al.* 1993a; Anderson and Gompels, 1999).

1.9.3 The association of glycoprotein H and glycoprotein L

Glycoprotein L has been identified as a chaperone for gH processing and transport to the cell surface in recombinant protein studies (Hutchinson *et al.* 1992; Yaswen *et al.* 1993; Pulford *et al.* 1995; Duus *et al.* 1995; Kaye *et al.* 1992; Liu *et al.* 1993b, Westra *et al.* 1997). Glycoprotein L homologs have been identified in all herpesvirus subfamilies and association with gH has been consistently reported (Duus *et al.* 1995; Hutchinson *et al.* 1992; Kaye *et al.* 1992; Liu *et al.* 1993b; Roop *et al.* 1993; Yaswen *et al.* 1993). Complex formation between gH and gL can be either non-covalent (HSV-1, VZV, EBV) or via disulfide bonds (CMV, HHV6) (Hutchinson *et al.* 1992); Duus *et al.* 1995; Kaye *et al.* 1992). The authentic expression of gH *in vitro* is dependent on this chaperone-like function of gL. A similar situation applies to gL expression itself, glycoprotein L is dependent on gH for its own post-translational modifications, since recombinant

expression studies of gL alone show that this glycoprotein is processed incompletely and either secreted into the culture medium (e.g. HSV) or retained within the cell (e.g. EBV) (Hutchinson *et al.* 1992; Roop *et al.* 1993; Spaete *et al.* 1993; Duus *et al.* 1995). Furthermore, in HSV-1 gL is not independently anchored to plasma membranes but is membrane associated only as a result of complex formation with gH (Dubin and Jiang, 1995). The protein encodes a N terminal signal sequence targeting the ER (Hutchinson *et al.* 1992) but lacks a membrane spanning domain. Although the family of gL proteins is divergent at the sequence level, a recent study showed that the gammaherpesvirus EBV gL and the alphaherpesvirus VZV gL can substitute functionally for each other in a transient transfection system (Li *et al.* 1997a). A similar observation has been reported for HHV6 and HCMV (Anderson *et al.* 1996). The way gH and gL interact is not yet clear. Within the N terminus of gH conserved cysteines have been identified within all herpesviral subfamilies, that may be required for binding (Duus *et al.* 1995; Anderson *et al.* 1996). How glycoprotein H in association with gL mediates membrane fusion is also not fully understood. Perhaps gL is only a chaperone that has no effect on fusion itself (Duus and Grose, 1996), or both gH and gL are co-localized at the surface and induce fusion in form of a complex (Peng *et al.* 1998b).

The gH/gL complex was also used in immunization studies of mice. Limited protection against viral challenge has been reported for VZV gH/gL antisera, where neutralizing, complement-independent, antibodies could be detected (Nemeckova *et al.* 1996). Also, using a secreted form of HSV-1 gH/gL where the transmembrane domain of gH had been removed, the sera of immunized mice contained high titers of

virus neutralizing antibodies (Peng *et al.* 1998a). However, in comparison to the highly protective glycoprotein D immunization, the immune response elicited by the complex was relatively inefficient (Browne *et al.* 1993).

Recently in some herpesviruses, including HCMV and EBV, a third viral glycoprotein has been associated with the gH/gL complex (Huber and Compton, 1997; Li *et al.* 1995). In EBV this third component, the protein gp42, plays a crucial role during viral entry into of B cells (Li *et al.* 1995). The existence of such a protein in KSHV has not yet been established.

1.9.4 A third component of the gH/gL complex

In the human cytomegalovirus three protein complexes, designated gCI, gCII and gCIII, have been identified that consist of disulfide-linked glycoproteins (Britt, 1984; Gretch *et al.* 1988). Complex gCI is composed of gB homodimers, gCII is defined as the gH/gL complex and gCIII is thought to contain gM (Britt, 1984; Cranage *et al.* 1988; Gretch *et al.* 1988; Kari and Gehrz, 1993). Recently a third component of gCIII has been identified, encoded by the HCMV UL74 ORF and designated as glycoprotein O (Huber and Compton, 1997; Li *et al.* 1997; Huber and Compton, 1998). The intracellular formation and processing of this tripartite complex is under investigation, but it has not been established how independent gH, gL and gO are for their processing and targeting (Huber and Compton, 1999). The function of gO remains to be analysed.

The gammaherpesvirus EBV is associated with a number of lymphoproliferative diseases. The virus is known to infect at least two different cell types in vivo, B lymphocytes and epithelial cells. However, in vitro it has only been possible to establish a productive EBV infection in B lymphocytes. Here, the virus attaches via the viral gp350 glycoprotein to CD21 and, in association with gH and gL, enters the host cell (Fingeroth *et al.* 1984; Nemerow *et al.* 1989; Haddad and Hutt Fletcher, 1989). Furthermore, a third protein gp42 associates non-covalently with the gH/gL complex (Li *et al.* 1995). This glycoprotein is encoded by the BZLF ORF and plays a critical role in the infection of B lymphocytes (Li *et al.* 1995). Monoclonal antibodies binding to gp42 inhibit fusion of the virus with the B cell membrane but have no effect on virus attachment to CD21 on B cells (Miller and Hutt Fletcher, 1988; Li *et al.* 1995; Wang and Hutt Fletcher, 1998). Reports have shown that gp42 binds to human leukocyte antigen (HLA) class II molecules to facilitate viral penetration (Li *et al.* 1997b; Spriggs *et al.* 1996). These class II molecules are found predominantly on antigen-presenting cells, such as macrophages, dendritic cells and B cells. Hence, glycoprotein gp42 probably represents an adaptation to infection of B lymphocytes, as gp42 is not involved in viral entry processes into epithelial cells (Li *et al.* 1995).

1.10 Project

The newly identified herpesvirus KSHV contains a large number of potential oncogenes and cellular homologs that may play a complex role in the development of this vascular sarcoma. However, a cell system to study viral entry as well as virus pathogenesis of KSHV has not yet been identified. This PhD thesis can be divided into three individual areas, each analysing a different feature of KSHV.

1.10.1 Characterization of ORF22 and ORF47, predicted to encode gH and gL

As described above, glycoprotein H is highly conserved within the family of herpesviruses and it is a major target of the host immune response. Its involvement in viral penetration into the host cell and the formation of syncytia is an interesting feature in identifying potential target cells. Glycoprotein H and glycoprotein L have been identified in KSHV as the gene products of ORF22 and ORF47 respectively. Analysis of their amino acid compositions showed similar features to other herpesviruses. Following mammalian expression of KSHV ORF22 and ORF47, either individually or in co-transfection experiments, their localization within the epithelial cell lines HEK293 and Cos-7 was determined. To facilitate the characterisation of the glycoproteins it was attempted to establish constitutively expressing cell lines. In summary, levels of expressed proteins were low in both cell lines and an in depth characterization was not possible because of the lack of glycoprotein-specific antibodies. However, the intracellular distribution of KSHV gL has been analysed and similar features to other herpesviruses demonstrated. Surprisingly, cell-to-cell fusion has also been detected when both cell lines, Cos-7 and 293, were transfected with gL alone.

1.10.2 A hepatoma cell line as a target for KSHV

The propagating of KSHV *in vitro* has been rather unsuccessful. B cell lines have been established from PEL patients and maintained in culture. However, these cell lines are already infected with KSHV, making them inadequate for *de novo* infections. The lack of such in vitro systems makes viral pathogenesis studies as well as the search for possible receptors used by KSHV complicated. Only recently, several research groups have identified cell lines that seem susceptible to KSHV infection and to support viral replication over an extended period of time.

During our research we identified the hepatoma cell line HepG2 to be able to support the growth of the KSHV positive B cell line BCP-1. Subsequent experiments showed that this cell line can be infected with KSHV under certain conditions. The presence of EBV in the inoculum was demonstrated to be necessary for KSHV to enter HepG2 cells successfully. In contrast, KSHV DNA could not be detected within HepG2 cells when incubated in the presence of cell-free supernatant from the KSHV singly infected BCP-1 cells. These results confirm an observation made by others, that EBV might be a supportive co factor in viral entry/growth under certain conditions (Kliche *et al.*, 1998).

1.10.3 Quantification of KSHV in plasma samples

Recently, the demand for quantification of pathogens, including viruses, bacteria, or fungus during disease progression has increased. Several assays have been developed that not only detect the infectious agent but also allow its quantification. This has the advantage that disease progression can be directly monitored and may help to assess the activity of antiviral drugs. Furthermore, these assays can be useful in

burden. KSHV infection of an individual can currently be demonstrated by the presence of antibodies using either serological assays or by PCR. As for KSHV and other herpesvirus, the viral life cycle can be divided into a latent and lytic stages. For research as well as for diagnostic purposes a quantitative assay may help to establish a correlation between the latent/lytic replication and disease progression by monitoring viral load.

The third part of this project describes the development of such a quantitative assay and testing of serum and plasma samples. Based on other viral load assays developed in the Department of Virology, a KSHV specific 96 well quantification assay was developed. Briefly, total DNA is extracted from patient serum samples or plasma samples, a specific sequence amplified by PCR, amplicons coated onto 96 well plates and, following hybridisation with a KSHV-specific probe, analysed by chemiluminescence. Several serum and plasma samples were tested and the data demonstrated (a) the assay is able to detect KSHV quantitatively and (b) that the virus is most prominently detectable in plasma, whereas serum shows a more than 10fold decrease in viral load.

2. Material and Methods

2.1 Material

All chemicals used throughout this thesis were obtained from Sigma or BDH unless otherwise stated. All solutions used are listed in Appendix I. Appendix II illustrates plasmid maps of all vectors used and Appendix III gives details about the manufacturers' addresses.

2.1.1 Plasmid Vectors

Original plasmids were resuspended in 1mM Tris-EDTA (pH 7.5) and stored at -20°C.

Plasmids were also transformed into *E.coli* on receipt and stored as glycerol stocks at -20°C.

pcDNA3: The vector pcDNA3 (Invitrogen) is designed for mammalian protein expression. It contains the cytomegalovirus (CMV) immediate early promoter for recombinant expression in eukaryotic cells as well as a T7 promoter/priming site, allowing the expression of the same protein in prokaryotic systems. For the establishment of a stable cell line the vector has a neomycin gene, enabling the transfected cells to grow in the presence of the antibiotic geneticin. Cloning procedures can be performed in E. coli as the vector carries a prokaryotic origin of replication (ColE1) and the ampicillin resistance gene. For a schematic map of the vector see Appendix II.

pcDNA3.3p6: This vector is a modified version of the plasmid pcDNA3. The CMV immediate early promoter as well as the T7 promoter/priming site were removed and replaced by the stronger CMV major immediate early enhancer-promoter sequence derived from the plasmid P2 (see below). Thus, this vector can offer advantages in the establishment of stable cell lines as the level of expression is increased. To further increase the efficiency of synthesis of mature recombinant glycoprotein, a 'leader sequence' encoding the first 21 amino acids of the tissue plasminogen activator (tPA) has been inserted downstream of the promoter. This allows cloning of DNA fragments immediately downstream of the leader sequence. The replacement of the natural signal sequence can result in more efficient post translational processing. Both, CMV promoter and tPA leader sequences were derived from the plasmid pEE14.gp120 (P2). The design and construction of pcDNA3.3p6 was carried out by Dr P Balfe and Dr J May.

P2 (pEE14.tPA-gp120) DNA sequences encoding HIV-1_{BH10} gp120 in frame with the signal sequence of the tissue plasminogen activator (tPA) have been inserted into the HindIII site of the multiple cloning side of pEE14.neo (Rhodes *et al.* 1994). The promoter is derived from the major immediate-early (MIE) gene enhancer-promoter of the human cytomegalovirus (hCMV). It contains the 5' untranslated sequence (intron) of the MIE gene (Accession number M21295; Akrigg *et al.*1985). The mammalian expression vector pEE14.neo was supplied by Celltech Research (kind gift by Dr Paul Stevens).

pcDNA3.1 This mammalian expression vector is an updated version of pcDNA3. It contains all features described for pcDNA3 to allow efficient protein expression, including CMV immediate early promoter, T7 promoter/priming site, origin of replication (ColE1), ampicillin resistance gene, etc.. To allow selection of positive transfectants the vector encodes the zeocin resistance protein (*Sh ble* gene).

The protein binds to zeocin and inhibits the antibotics DNA strand cleavage activity, hence allowing cellular growth in the presence of zeocin. The multiple cloning site of pcDNA3 was extended and slightly modified to disrupt a formation of an mRNA hairpin structure which has been demonstrated to affect recombinant expression of genes cloned downstream of the restriction site NotI (Invitrogen, personal communication).

pcDNA3.1-β- Gal-His This mammalian expression vector was kindly provided by Invitrogen (Leiden, The Netherlands) and encodes a histidine tagged β-galactosidase. The vector is used in optimization studies of transfection and western blot procedures.

2.1.2 Prokaryotic cells

Bacterial strains were grown aerobically at 37°C in an orbital shaker. For plasmid purification purposes 3ml (Miniprep) to 25ml (Midiprep) of Luria Bertani (LB) medium supplemented with 100μg/ml ampicillin were inoculated with a fresh over-night culture. E. coli *SCS-1* and *XL1-blue* were obtained from Stratagene as

bacterial stabs. Full genotypes of these strains are given in the company's catalogue.

E coli SCS-1 This bacterial strain is *recA* deficient to ensure insert stability and has been shown to reliably yield good competent cells. Competent bacteria are used in DNA cloning procedures. Prior to transformation cells were treated with calcium chloride to allow subsequent uptake of plasmid DNA.

XL-1 blue This bacterial strain contains an antibiotic (Tetracyclin) resistant F' episome. For high quality plasmid DNA it has a mutation in the endA1 gene. E. coli XL1-blue is used in DNA cloning procedures as described for the SCS-1 strain.

2.1.3 Mammalian cell lines

All mammalian cell lines were maintained in T75 or T25 sterile tissue culture flasks and grown at 37°C in a 5% CO_2 humid atmosphere.

293 cells: The HEK 293 cell line is derived from human embryonal kidneys and is used in the recombinant expression of glycoproteins. 293 cells were transformed to continuous growth using adenovirus Ad5 DNA.

Morphology: Epithelial

Media: Dulbecco's Modified Eagles Medium (DMEM) (ICN) supplemented with 10% (v/v) Foetel Calf serum (FCS),

2mM L-glutamine, 50 IU/ml penicillin, 50mg/ml streptomycin and 0.075% sodium bicarbonate.

COS-7: Cos-7 cells are derived from african green monkey kidneys. Cells were transformed with an SV40 mutant, which encodes for the large T-Antigen. Cos-7 cells are used in combination with the mammalian expression vector pcDNA3.1. The vector encodes for SV40 origin of replication and therefore is capable of episomal replication in this cell line.

Morphology: Fibroblasts

Media: RPMI with GlutamaxII (Life Technologies), supplemented with 10% (v/v) Foetel Calf serum (FCS), 50 IU/ml penicillin, 50mg/ml streptomycin and 0.075% sodium bicarbonate

HepG2 cells HepG2 cells are a well differentiated liver cell line, exhibiting biosynthetic capabilities of normal liver parenchymal cells. Originally established from a 15 year old male Caucasian, this hepatoma cell line was used in infection studies of KSHV.

Morphology: Epithelial

Media: RPMI with GlutamaxII (Life Technologies), supplemented with 10% (v/v) Foetel Calf serum (FCS), 50 IU/ml penicillin, 50mg/ml streptomycin and 0.075% sodium bicarbonate

BCP-1 cells: BCP-1 cells are derived from the peripheral blood of a patient with body cavity based lymphoma. Cells are singly infected with HHV8 and lack *c-myc* rearrangements (Boshoff *et al.* 1998). Following several experiments BCP-1 cells were maintained when co-cultivated with human embryonic lung (HEL) cells as feeder cell monolayer. BCP-1 cells were obtained from the Institute of Cancer Reasearch, London, UK.

Morphology: B cell lineage

Media: RPMI with GlutamaxII (Life Technologies), supplemented with 10% (v/v) Foetel Calf serum (FCS), 50 IU/ml penicillin, 50mg/ml streptomycin and 0.075% Sodium bicarbonate

SY cells SY cells are derived from primary effusion lymphoma and the cell line was established in the Virology Department, Windeyer Building, RFUCMS, UCL, London, UK. Cells are dually infected with KSHV and EBV (Ursula Ayliffe, personal communication). SY cells are maintained in the presence of a feeder layer of human embryonic lung (HEL) cells.

Morphology: B cell lineage

Media: RPMI with GlutamaxII (Life Technologies), supplemented with 10% (v/v) Foetel Calf serum (FCS), 50 IU/ml penicillin, 50mg/ml streptomycin and 0.075% sodium bicarbonate

2.1.4 Antibodies

α-His-IgG1 Monoclonal Antibodies

Mouse Monoclonal antibodies detecting proteins with a histidine tag were obtained from Qiagen. The Penta-Histidine monoclonal antibody recognizes five consecutive histidine residues, whereas the Tetra-histidine monoclonal antibody is highly specific for four consecutive histidine residues.

α-c-myc-Monoclonal Antibody

A hybridoma cell line producing the monoclonal antibody 9E10 was kindly provided by the Imperial Cancer Research Fund. The antibody is used in the detection of recombinant proteins containing the c-myc epitope.

Epitope: EQKLISQQDL

Goat α-mouse IgG Horse Radish Peroxidase conjugate

This antibody is used as a secondary antibody, in Western Blot procedures and was supplied by Harlan Sera-Labs.

Goat α-mouse IgG FITC conjugate

This antibody is used as a secondary antibody in indirect immunofluorescence studies and FACScan analysis (supplied by Dako).

Goat α-mouse IgG β-Galactosidase conjugate

This antibody is used as secondary antibody in histochemical staining procedures and was supplied by Harlan Sera-Labs.

2.1.5 Chemicals used in staining of Golgi organelles

The chemical probe BODIPY-TR were obtained from Molecular Probes (Leiden, The Netherlands) and used as indicated in the manufacturer's instructions.

2.1.6 Enzymes

All reactions requiring enzymes were performed following the manufacturers guidelines.

Enzymes	**Manufacturer**
Restriction endonucleases	Roche Diagnostics (Boehringer Mannheim)
T4 DNA Ligase	Promega
TaqGold DNA polymerase	Perkin Elmer
Taq DNA Polymerase	Promega
Expand Long Template DNA Polymerase	Roche Diagnostics (Boehringer Mannheim)
Uracil DNA Glycosylase	Roche Diagnostics (Boehringer Mannheim)
Shrimp Alkaline Phosphatase	Amersham (USB)

2.1.7 Oligonucleotides

The design of oligonucleotides is described for each polymerase chain reaction individually. All primers were obtained from Oswel DNA Service, Southampton, UK.

2.2 Methods

2.2.1 Patient samples

Patient samples were obtained from the UCH Diagnostic Virology Service as serum and plasma. Samples were stored at -20°C and for DNA extraction thawed quickly in a 37°C water bath. These were residual samples remaining after diagnostic testing. Consent was obtained from the chairman of the UCLH Ethics Committee for use of these samples (see attached letter, Appendix IV). All samples were tested anonymously.

2.2.1.1 Sample preparation

EDTA blood was centrifuged for 5min at 1800rpm and 1ml of plasma transferred to a 1.5ml screw top eppendorf tube. An equal volume of PBS was added to the remaining blood, the tube inverted and 1ml of reconstituted blood collected and stored in 1.5ml screw top eppendorf tubes. Serum, plasma and whole blood were stored at -20°C in accordance with the standard laboratory practice in the Diagnostic Virology laboratory at UCLH.

A variety of clinical samples, as well as cell culture material were used as DNA templates for the amplification of specific viral sequences. Total genomic DNA was prepared using a blood extraction kit, obtained from Qiagen, in accordance with the manufacturers instructions.

2.2.1.2 QIAamp blood Mini kit total DNA extraction

Total DNA purification from cell culture material and patient blood or plasma was performed according to the instructions supplied with the QIAamp blood Mini kit (Qiagen). Briefly, 200µl of the sample were mixed with 200µl of lysis buffer, containing Proteinase K (10µl/ml). After 10 min incubation at 56°C, 200µl absolute ethanol was added, tubes vortexed for 15 sec and the mixture applied to a spin column. Following three spin-wash steps the DNA was eluted in 50µl of elution buffer and the extracted DNA stored at -20°C.

2.2.1.3 DNA from cell culture supernatant containing infectious viral particles

KSHV DNA was prepared from BCP-1 and SY cell culture supernatants. Supernatant was collected in 1.5ml screw top eppendorf tubes and centrifuged for 5min at 5000rpm to remove any remaining cells. After 5 min boiling eppendorf tubes were wiped down with ethanol and removed from the category 3 laboratory. Total DNA was then extracted using the Qiagen Blood Mini Kit and the viral copy number determined by Poisson limited dilution PCR. Twenty microliter aliquots were stored at -20°C.

2.2.2 Polymerase Chain Reaction (PCR)

The polymerase chain reaction was applied at several stages of this thesis. Firstly for the amplification of viral DNA sequences encoding glycoprotein H and glycoprotein L (Cloning PCR), secondly to test for the presence of KSHV DNA in the hepatoma

cell line HepG2 (Infection PCR) and finally for the quantification of KSHV in patient blood samples (ELONA-PCR).

2.2.2.1 Sequences and Primer design

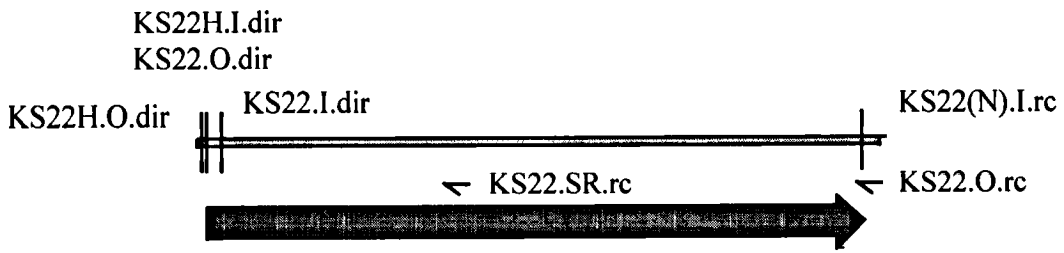
A complete list of all oligonucleotide sequences used in this thesis is given on the following pages (table 2.1 to 2.3). In addition schematic pictures illustrate their localization within the viral open reading frames.

2.2.2.1a Cloning PCR

The DNA sequences as well as the amino acid sequences encoding the viral glycoproteins were obtained from GenBank (Accesion number: U75698). Analyses to determine the immunoreactivity of each protein were performed using the PREDICT program of the G-C-G package. Signal sequence cleavage sites were predicted for each protein using the SignalP V1.1 World Wide Web Server (Nielsen *et al.* 1997).
The KSHV open reading frames 22 and 47 were amplified by nested polymerase chain reaction and inserted into mammalian expression vectors. Synthetic oligonucleotide primers were designed depending on the vector of choice.

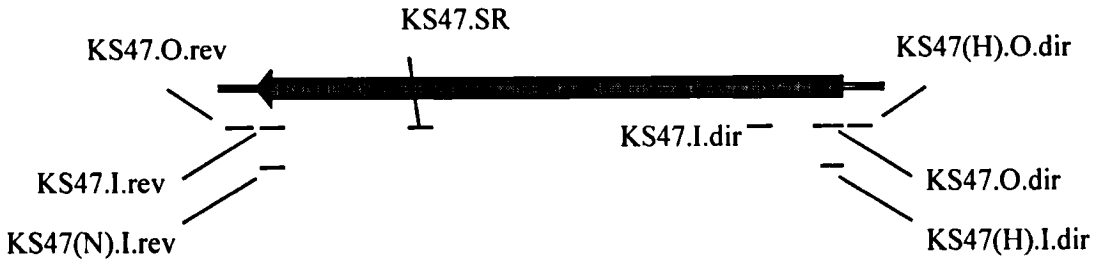
Primers for the amplification of truncated protein

The vector pcDNA3.3p6 encodes the signal sequence, amino acid 1 to amino acid 21, of the tissue plasminogen activator (tPA). Viral glycoproteins were therefore



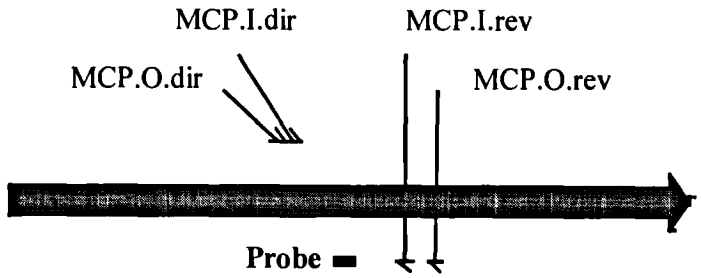
Oligonucleotide primer (Oswel code numbers)	Sequence (5`-3`)	Localization	Target Vector
KS22H.O.dir (A 4227)	GCAGTTAATCACCTAGAG	37090-37107	pcDNA3.1
KS22.O.dir (V 8673)	*ATGCAGGGTCTAGCCTTC*	37113-37130	pcDNA3.3p6
KS22.O.RC (V8674)	CTACGCTTTATTGACCGT	39323-39305	pcDNA3.3p6 pcDNA3.1
KS22H.I.dir (A4228)	CGCGCCGTACGAAGCTT**GCCACC***ATGCAGGGTCTAGCCTTC*	37113-37130	pcDNA3.1
KS22.I.dir (V8675)	AAUUGAAGGUCGU*TTGACATGTGGAGCCACTG*	37164-37182	pcDNA3.3p6
KS22N.I.RC (A4229)	AGACTCGAGCGGCCGCCATTA*ATGATGATGATGATGATGATAAAGGATGGAAAACAG*	39300-39281	pcDNA3.1
KS22.I.RC (V8684)	UUA*AUGAUGAUGATGATGATGATAAAGGATGGAAAACAGTCTG*	39300-39281	pcDNA3.3p6
KS22.SR (V8676)	GAAATATGCGAGTGTAATTCTC	37929-37908	Colony screen gH

Table 2.1: Oligonucleotide primers for the amplification of KSHV ORF22 (glycoprotein H). Localization is given with respect to the DNA sequences published by Russo *et al.* 1996. Italic Letters: KSHV ORF22 sequences; Underlined letters: Restriction sites; Bold black letters: Kozak sequence; Italic, underlined letters: 6xHis protein tag (anti codons shown)



Oligonucleotide primer (Oswel code numbers)	Sequence (5`-3`)	Localization	Target vector
KS47H.O.dir (A4237)	CAAGGGTGAAACCCGGAT	69939-69922	pcDNA3.1
KS47.O.dir (W6685)	*ATGGGGATCTTTGCGCTATTTG*	69915-69899	pcDNA3.3p6
KS47.O.RC (W6686)	GTTGCAACCATGCGTCCATG	69386-96405	pcDNA3.3p6 pcDNA3.1
KS47H.I.dir (A4238)	CGCGCCGTACGAAGCTT**GCCACC***ATGGGGATCTTTGCGCT*	69899-69915	pcDNA3.1
KS47.I.dir (W6687)	AAUUGAAGGUCGUTACGTCGCCTTACCATGTTG	69855-69836	pcDNA3.3p6
KS47N.I.RC (A4236)	AGACTCGAGCGGCCGCC*ATTAATTCAGATCCTCTTCTGAGATGAGTTTTTGTTCTTTTCCCTTTT GACCTGCG*	69415-69433	pcDNA3.1
KS47.I.RC (W6694)	UUAAUGAUGAUG*TTACAGATCCTCTTCTGAGATGAGTTTTTGTTCTTTTCCCTTTTGACCTGCG*	69415-69433	pcDNA3.3p6
KS47.SR (W6688)	AGCTATCTGCAGTGGTCG	69543-69560	Colony screen gL

Table 2.2: Oligonucleotide primers for the amplification of KSHV ORF47 (glycoprotein L). Localization is given with respect to the DNA sequences published by Russo *et al.* 1996. Italic letters: KSHV ORF47 sequences; Underlined letters: Restriction sites; Bold letters: Kozak sequence; Italic, underlined letters: c-myc protein tag (anti codons shown)



Oligonucleotide primer (Oswel code numbers)	Sequence (5'-3')	Localization	Application
MCP.O.dir (S2916)	AGCCGAAAGGATTCCACCAT	47297-47306	Infection and ELONA PCR
MCP.O.RC (T0872)	TCCGTGTTGTCTACGTCCA	47519-47502	Infection and ELONA PCR
MCP.I.dir (F3647)	GTGCTCGAATCCAACGGATT	47308-47327	Infection and ELONA PCR
MCP.I.RC (T0874)	ATGACACATTGGTGGTATAT	47479-47460	Infection and ELONA PCR
MCP.probe (S2917)	ACACCAACAGCTGCTGC	47395-47379	Infection and ELONA PCR

Table 2.3: Oligonucleotide primers for the amplification of a region within HHV8 ORF26 (Minor Capsid Protein, 912bp). Localization is given with respect to the DNA sequences published by Russo *et al.* 1996

amplified without their own signal sequence in order to be expressed in frame with the tPA leader sequence. The sense primers used in the first round of PCR are located at the 5'end of the coding sequence whereas the 3' oligonucleotides are complementary to sequences outside the coding region. To increase cloning efficiency the set of inner primers were designed with uracilated tails as described in the Clone Amp™ pAmp1 rapid Cloning system by Life Technologies. These cloning 'tails' were complementary to sequences annealed to the linearised pcDNA3.3p6 vector, allowing cloning in the presence of the enzyme Uracil DNA glycosylase (J May Ph.D. thesis, 1998).

In addition second round primers encoded the restriction sites EcoRI (sense primer) and NotI (anti-sense primer) to facilitate possible subcloning. For protein detection purposes the anti-sense inner primer encoded either a C-terminal c-myc motif, for glycoprotein L constructs, or in case of glycoprotein H, six histidine residues, together with a stop codon for proper termination.

Primers for the amplification of the entire coding sequence

For the amplification of full-length sequences encoding the viral glycoproteins, a separate set of primers was designed. Sense and anti-sense primers used in the first round of PCR were both localized outside the coding region. Inner primers were designed to anneal to the 3' and 5' ends of the protein coding region. As described above inner primers also encoded restriction sites, HindIII and NotI, as well as C

terminal protein tags. Amplicons were inserted into pcDNA3.1 by HindIII/NotI restriction digest followed by ligation.

2.2.2.1b Infection PCR and ELONA-PCR

Infection and ELONA PCR are based on oligonucleotide primers specific for KSHV ORF26. Patient samples and tissue culture material were tested for the presence of KSHV by nested PCR. However, to quantify samples a single round PCR was applied.

Primers were kindly provided by Dr. M Howard. The primers were specific for sequences within the KSHV ORF26. The first round primer set has been published in 1994 by Chang and colleagues and were used in the initial identification of KSHV. For nested PCR an inner primer pair has been designed as detailed in Dr. M Howards' Ph.D. thesis (1999). For the enzyme linked oligonucleotide assay (ELONA) an anti-sense oligonucleotide has been designed which is complementary to sequences within the second round PCR product. This probe is 5' alkaline phosphatase conjugated and used for the quantification of KSHV in patient samples.

For optimal primer design sequence variability between different KSHV isolates sequences was analysed by alignments of all known viral ORF26 sequences using Clustal.W. Sequences were obtained from GenBank.

2.2.2.2 PCR parameters

The polymerase chain reaction conditions for the amplification of each DNA sequence was optimized individually. Different parameters, such as annealing temperature, oligonucleotide primer concentrations, amount of template as well as the quantity of DNA polymerase were assessed for each DNA template. Prior to amplification all PCR reactions were overlaid with 50µl paraffin to prevent evaporation.

2.2.2.2a Cloning PCR

Using a RoboCycler thermal cycler (Stratagene) optimal annealing temperature, oligonucleotide primer concentration and cycle count for the amplification reaction of KSHV ORF22 and ORF47 were assessed. Once an optimal amplification was achieved subsequent reactions were carried out using Perkin Elmer TC-1 thermal cyclers.

To avoid possible misincorporation during the amplification reaction the Expand Long Template DNA Polymerase was used. This mixture of proof reading (Pwo DNA polymerase) and non-proof reading (Taq DNA polymerase) DNA polymerases allows the efficient amplification of long DNA templates with high fidelity.

Amplification of ORF22, encoding glycoprotein H

For the amplification of KSHV glycoprotein H DNA samples were applied to two round-nested PCR, each round comprising 30 cycles, followed by a 7min extension at 72°C.

The first round reaction was performed using a 50µl reaction mixture containing 1xExpand PCR reaction buffer 3, 400µM dNTPs, 1U Expand DNA polymerase and the oligonucleotide primer pair KS22.O.dir (or KS22H.O.dir) and KS22.O.RC at a final concentration of 200µM. Two microliters of this first round product was used as the DNA template in the second round of the nested PCR. Amplification was achieved using 1xExpand PCR reaction buffer, 400µM dNTPs, 1U Expand DNA polymerase and the oligonucleotide primer pair KS22.I.dir (or KS22H.I.dir) and KS22.I.RC (or KS22N.I.RC) at a final concentration of 100µM. Cycling conditions for both rounds are as follows: Initial denaturing for 4 min at 94°C, 30 cycles of 45 sec at 94°C, 45 sec at 54°C and 3 min 30 sec at 68°C.

Amplification of ORF47, encoding glycoprotein L

KSHV ORF47 was amplified using the primer set KS47.O.dir/KS47H.O.dir and KS47.O.RC during the first round, and primer set KS47.I.dir/KS47H.I.dir and KS47.I.RC/KS47N.I.RC in the second round. DNA was amplified using the same reaction conditions as described above for ORF22, except primers were used at a final concentration of 100µM, respectively.

2.2.2.2b Infection PCR

Reaction conditions were initially developed by Dr. Mark Howard but optimised in this thesis.

Total DNA from tissue culture material was purified using the Qiagen Mini Blood kit. One to ten microliters of extracted DNA were transferred to a 50µl PCR mix, containing 1xTaq reaction buffer, 1.5mM $MgCl_2$, 1mM dNTPs, 1.5U Taq DNA polymerase and the outer primers MCP.O.dir and MCP.O.rev at a final concentration of 200µM. The mixture was subjected to an initial denaturing step at 95°C for 4 min followed by 35 cycles (94°C, 1 min; 50°C, 1 min; 72°C, 1 min) of amplification and a final extension at 72°C for 7 min.

One microliter was transferred to 25µl of a second round PCR mix. Amplification was achieved using 1xTaq reaction buffer, 1mM dNTPs, 1U Taq DNA Polymerase and the primers MCP.I.dir and MCP.I.rev at a final concentration of 500µM and the cycling conditions 4 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C and finally 7 min at 72°C. Ten microliters of PCR product were visualized by electrophoresis on a 1% agarose gel.

2.2.2.3 Limit Dilution Polymerase Chain Reaction

If the PCR used is "limit sensitive", i.e. capable of detecting a single molecule of DNA, the number of viral DNA molecules within a DNA sample can be assessed by a limit dilution polymerase chain reaction. The DNA amplification performed as detailed for the infection PCR was shown to be limit sensitive.

In general the limit dilution PCR was performed in sets of ten tubes. Each member of the set containing the same dilution of the sample. Ten microliters of the neat or diluted sample were used in the nested PCR described above. Following the reaction ten microliters of each sample were analysed by gel electrophoresis and the number of viral DNA molecules within the tested sample was calculated as follows: the number of negative or 'zero' (f_0) amplification products in each set of 10 tubes was counted. According to the zero term of the Poisson probability distribution the average number of DNA molecules per tube (μ) can be assessed by inserting the frequency of non-amplifications per set into the following equation:

$$\mu = - \ln (f_0)$$

Taking the dilution factor into account the number of DNA molecules per sample can then be determined. For example if 5/10 reactions were negative $f_0 = 0.5$ and $\mu = 0.69$, taking the dilution factor into account the copy number can be determined by simple multiplication.

2.2.3 Enzyme linked oligonucleotide assay (ELONA)

2.2.3.1 ELONA-PCR

To determine the number of KSHV copies within DNA samples from patients, a single round PCR was performed. The ORF26 has been analysed for sequence variation between viral isolates (Zong *et al.* 1997). The second round primer set was chosen for the ELONA-PCR because more sequence data was available, indicating a high level of sequence conservation.

One and 10 microliters of Qiagen extracted DNA from patient blood samples was subjected to the second round of the above described nested PCR (infection PCR). The volume of reaction was increased to 50µl per tube and 38 cycles of PCR performed. Optimization experiments showed this number of cycles to give the best result (see chapter six). Following the reaction 5µl of the PCR product was analysed by an enzyme linked oligonucleotide assay.

2.2.3.2 ELONA

Whitby and Garson originally described this assay in 1995. Briefly, a biotinylated PCR product is captured onto a blocked streptavidin coated plate, the dsDNA denatured using NaOH and an alkaline phosphatase conjugated oligonucleotide (probe) added. An enzymatic reaction with a luminescent phosphatase substrate (Lumiphos) can be measured by a luminometer in relative light units (RLU). Black 96 well streptavidin coated plates were purchased from Advanced Biotechnologies (see Appendix III). Single round PCR products were diluted one in twenty in sample

diluent (see Appendix I) and 100µl per well added. After 45min incubation at 45°C unbound PCR product was washed away using a plate washer, washing each well with 1xTTA (see Appendix I) 10 times followed by a 1min soak. To denature the dsDNA each well was incubated with 100µl of 0.15M NaOH for 5min. After another wash step, 100µl of alkaline phosphatase conjugated internal probe, diluted 1 in 1800 (2.7nM f.c.) in probe diluent (see Appendix I) was added to each well. The probe was left to anneal for 30min at 45°C. The plate was then washed three times as described above, leaving it to soak for 1min after each wash. Subsequently Lumiphos (100µl/well) was added, the plate sealed and incubated for 1h in the dark to avoid photobleaching. After one hour incubation light emission reaches a plateau level and measurements can be performed.

The emitted photons were measured using a TopCount 480 luminometer (Packard). Data was analysed using Genesis Version 2.20 software.

2.2.4 DNA analysis and manipulation

2.2.4.1 DNA electrophoresis

DNA electrophoresis was performed using agarose as a matrix, generally at a concentration of 1% (w/v) dissolved in 1xTAE. The DNA fragments were directly visualized by ethidium bromide added to the agarose gel (0.2µg/ml). Samples were diluted in loading dye and 12µl per well loaded. To estimate the size of the DNA

fragments a 1kb DNA ladder (Life Technologies) was also loaded in each gel. The gel run was generally performed at 100V and 80mA for 45min.

2.2.4.2 Purification of DNA fragments from agarose gels using the GenecleanII Kit (BIO 101 Inc).

DNA was separated on 1% agarose gel and the desired band excised using a sterile scalpel blade. The gel block was transferred to a plastic 1.5ml eppendorf tube and the DNA purified following the instructions of the manufacturer. The extraction kit is based on a specially formulated silica matrix (glassmilk) which binds the DNA. Following three washes of the DNA-glassmilk complex the DNA was eluted in 20µl TE by incubating the tube at 50°C for 3 min.

2.2.4.3 Restriction Endonuclease Digest

Restriction digest procedures were used in cloning procedures and to confirm the cloned insert. Restriction enzymes were used in combination with the reaction buffer recommended by the manufacturer. For NotI/HindIII restriction digests buffer H was used (Roche) (see Appendix), for EcoRI/XhoI digests the reaction was performed in 1xUniversal buffer (Stratagene) (see Appendix I).

In general 250 to 400ng of purified plasmid DNA were mixed with 10-16U of restriction enzyme, 1xReaction Buffer, and water to a final volume of 20 to 30 µl. The reaction mixture was incubated for 37°C for 2h. To inactivate enzyme activity an

incubation of 15 min at 65°C was performed. The resulting DNA fragments were analysed on a 1% agarose gel.

2.2.4.4 Dephosphorylation of linearised vector

For cloning procedures the linearised expression vector was gel purified and dephosphorylated to prevent self-ligation. One unit of Shrimp alkaline phosphatase and 1xReaction buffer were added to the purified and digested DNA. Following a 20 min incubation at 37°C, the phosphatase was inactivated for 15min at 65°C.

2.2.4.5 Ligation and Annealing reaction

2.2.4.5a Ligation

During the ligation reaction the linearised vector and the insert of interest are mixed together and ligated by the action of the enzyme T4 DNA ligase. The plasmids pcDNA3 and pcDNA3.1 were digested with the restriction enzymes *HindIII* and *NotI*. The insert, encoding the desired protein, was prepared with the same restriction enzymes as the target vector. The PCR products were purified by agarose gel electrophoresis and restriction digested. To eliminate the restriction enzymes 5µl of Strataclean resin was added and, after a five minute incubation at room temperature, pelleted at 13000rpm for one minute. 200-250ng of the pre-treated linearised vector was used per ligation reaction. The amount of insert was calculated individually. For

the cloning of glycoprotein H a vector:insert ratio of 1:2 was successful, as was a 1:8 ratio for glycoprotein L.

The entire 20µl ligation mix contained the above mentioned components as well as 1xLigase Buffer and 3U of T4 DNA ligase. Digested vector without insert served as a negative control. Ligations were performed over night at 12°C.

2.2.4.5b Cloning into pcDNA3.3p6

The Clone Amp rapid cloning system by Life Technologies was modified for the pcDNA3.3p6 vector. Double stranded tails that consisted of an uracilated strand and a complementary dTMP strand were ligated to the linearised vectors. As described earlier the insert was amplified with primers encoding complementary uracil-containing extensions. In the presence of the enzyme Uracil DNA Glycosylase these uracilated bases will be removed, producing sticky ends. Vector and insert will then anneal, resulting in a circulised form of the vector, which can be transformed into competent bacteria (J May PhD thesis, 1998). The vector pcDNA3.3p6 was kindly provided in linearised and tailed form by Dr J May.

Tails were designed to create an EcoRI and Not I restriction site at the 3' and 5' end of the insert, allowing future subcloning of the insert. PCR products were purified by agarose gel electrophoresis and 7-10µl (100-150ng) added to a 20µl reaction mix containing 1xAnnealing Buffer, 25ng of the vector and 1U UDG. The annealing

reaction took place at 37°C for 1h and 10µl of the mixture were transformed into calcium competent *E.coli* SCS-1.

2.2.4.6 Transformation

2.2.4.6a Competent Bacteria

A 250ml vented Erhlenmeyer flask containing 50ml LB growth medium was inoculated with 500µl of an *E. coli* SCS-1 or *E coli* XL-1blue over night culture and incubated at 37°C in an orbital shaker. Flasks were grown to mid log phase and subsequently placed on ice for 15min. Following a five minute spin at 3000rpm in a pre cooled (4°C) centrifuge the supernatant was removed and the pellet resuspended in 100mM ice-cold calcium chloride (25ml). After a further 1.30h incubation on ice, bacteria were centrifuged as before and again resuspended in 100mM calcium chloride (5ml). All steps were performed on ice to maintain cell viability.

2.2.4.6b Transformation

100-200µl of competent *E. coli* were transferred to round bottom transformation tubes (Falcon 2059) and placed on ice. Five to eight microliters of the ligation mix (10µl of the annealing reaction) or 250ng of purified plasmid DNA was added. A 30-40 min incubation on ice allowed the adsorption of the plasmid to the bacterial surface. Subsequent transfer to a 42°C preheated water bath for 90sec resulted in the uptake of DNA by the competent bacteria. After a short recovery on ice, the bacteria were

spread on LB/ampicillin (f.c. 100µg/µl) plates and the dishes incubated overnight at 37°C. Colonies were screened by PCR to verify the presence of inserts.

2.2.4.7 Screening of bacterial transformants by PCR

Colonies obtained were screened by a single round PCR for the presence of gH and gL. To facilitate the amplification 'screening' primers (KS22.SR and KS47.SR) were designed, which produce small DNA subfragments of the insert.

Using a flamed loop single colonies were transferred to a numbered grid on an ampicillin agar plate as well as added to a prepared 20µl PCR mixture containing 1 U Taq DNA polymerase, 1xTaq reaction buffer, 1.5mM $MgCl_2$, 100µM of each dNTP and the appropriate synthetic oligonucleotide pairs at a final concentration of 100nM each (KS22.I.dir/KS22.SR or KS47.I.dir/KS47.SR).

Targets were amplified in a 25 cycle PCR (94°C, 35sec, 35sec, 50°C, 2min 30sec, 72°C) followed by an extension of 7min at 72°C. Ten microliters of the reaction were analyzed by agarose gel electrophoresis.

2.2.4.8 Small Scale Plasmid preparation provided by QIAprep Plasmid Kit

PCR positive colonies were grown aerobically overnight in 3ml LB-Medium, containing 100µg per ml ampicillin for selection. The following morning plasmids were purified according to the manufacturers instructions (Qiagen). The QIAprep Kit uses solid-phase anion exchange separation method.

Cells were pelleted and an alkaline lysis performed. Proteins, cell debris and chromosomal DNA were precipitated with SDS and high salt, whereas the supercoiled plasmid DNA renatured and remained in solution. Subsequent centrifugation for 10min at 16000rpm separated the two fractions and the supernatant was bound onto a Qiagen gravity flow column. After two wash steps the DNA was eluted with Elution Buffer, supplied with the Kit. Following isopropanol precipitation the DNA was washed in 70% ethanol and finally dissolved in 100µl TE. The average yield was between 150-200ng per microliter. The purified plasmids were analyzed by restriction digest to verify the cloning procedure. Bacterial clones containing the plasmid of interest were grown over night in 3ml LB/ampicillin and an aliquot stored as a glycerol stock (500µl bacterial culture mixed with 500µl of 98% glycerol) at -20°C. Bacteria grown from glycerol stocks were streaked onto LB agar plates supplemented with 100µg/ml ampicillin and incubated over night at 37°C. The following morning a single colony was transferred to 3ml of selective growth media and incubated for 8h at 37°C. This starter culture was then diluted 1 in 800 and grown over night for further plasmid purification.

2.2.4.9 Midi DNA plasmid purification

For high yield plasmid purification, the Qiagen Midi Kit, providing gravity flow columns with a higher DNA binding capacity, was used. A single colony was transferred to selective LB medium and cultured for 8h at 37°C, Following a 1 in 800

dilution bacteria were incubated for an additional 16 to 18h in 25ml LB-Medium, containing 100µg per ml ampicillin for selection. The DNA purification was performed according to the manufacturers protocol. The pellet in the final step was eluted in 300µl TE, the average yield was approximately 350-700ng per microliter.

2.2.5 Automated Sequencing

Plasmids were sequenced using the Thermo Sequenase pre-mixed cycle sequencing kit (RPN 2444) and the Vistra DNA Sequencer 725 by Amersham.

The sequencing protocol is based on repeated cycles of thermal denaturing, annealing and extension/termination. DNA strand synthesis starts at a specific site, as determined by the Texas Red labelled primer, and ends with the incorporation of a chain terminating dideoxynucleoside triphosphate.

DNA samples were sequenced using the sense primer pCMV and the antisense primer p3rev. Oligonucleotide sequences are given in table 2.4.

Oligonucleotide primer (Oswell code numbers)	Sequence (5'-3')	Localization
pCMV	CGCAAATGGGCGGTAGGCGTG	CMV promoter
p3rev	TAGAAGGCACAGTCGAGG	BGH polyadenylation signal

Table 2.4: Oligonucleotide primers used in sequencing of plasmids. Localization is given with respect to the DNA sequences published by Invitrogen.

Cycle sequencing protocol

Plasmids were prepared using the Qiagen Plasmid Mini Kit. A 25µl master mix was prepared containing 2pmol Texas Red primer and 900ng of plasmid DNA. Six microliter of this template/primer mixture was transferred into four eppendorf tubes, already containing 2µl of either A, C, G or T reagents. Prior to PCR the reactions were overlayed with 20µl of paraffin. Tubes were placed into a Perkin Elmer Thermal Cycler and received an initial 4 min denaturing step at 94°C, followed by 25 cycles of 94°C for 15 sec, 50°C for 15 sec and 72°C for 30 sec.

Following amplification samples were transferred to a 96 well microtitre plate and mixed with 3µl of loading dye. The plate was then placed in a vacuum desiccator on a hot (80°C) aluminium block and left to evaporate for approximately 10 to 15min to reduce the volume to 3µl. Samples were loaded on the Vistra 750 sequencer. A mixture of loading dye, salt solution and water (2:1:1) was loaded into all empty lanes to avoid thermal artifacts.

Sequencing gels contained 11g urea diluted in 3mls 10xTBE, 3mls 2%BIS/40% Acrylamide (Sigma), 1ml 40% Acrylamide and 15ml of water. For polymerization 200µl of APS and 20µl of TEMED were added. Generally sequencing gels were run for 9h at 1400V

Computer analysis

Sequences were analysed using Sequencher 3.1. (Gene Codes Corp.).

2.2.6 Mammalian Cell culture

2.2.6.1 Maintenance of mammalian cell lines

In general all cell lines were maintained in T75 tissue culture flasks and passaged twice a week in a one to eight ratio. Culture media was removed and cells washed once in 4ml PBS to remove residual media. Cells were then covered with 2ml of Trypsin/EDTA. Following a 5min incubation at 37°C the flask was shaken until the cells were in suspension. An appropriate volume of cells was then transferred to a new T75 tissue culture flask, already containing 15ml of complete growth medium (see Appendix I).

Established stable cell lines were maintained in complete media supplemented with the appropriate drug and maintained as described above (see Appendix I).

2.2.6.2 Cryogenic preservation of mammalian cell lines

Transfected cells acquiring resistance to neomycin or zeocin were selected. Prior to single cell cloning, nitrogen stocks were prepared. A 90% confluent T75 flask was harvested using Trypsin/EDTA and cells were pelleted at 1500rpm for five minutes. Supernatant was removed and the cells were resuspended in 4ml of freezing media (see Appendix I). This mixture was transferred to four pre-cooled Nunc Cryovials and cooled slowly over night to -80°C before storage in a liquid nitrogen container.

To recover cells from nitrogen stocks, vials were rapidly thawed in a 37°C waterbath, diluted into 10mls complete media, and subsequently centrifuged for 5min at 1500rpm. Supernatant was removed and cells resuspended in 15ml of pre-warmed

complete or selective growth media. The resuspended cells were then transferred to T75 tissue culture flasks. Media was replaced the following day.

2.2.6.3 Transfection

Different methods of transfecting mammalian cells have been tested. The efficiency of transfection was assessed using the plasmid pcDNA3.1-betaGal-His (see Appendix II for plasmid map).

2.2.6.3a LipofectAMINE™ Transfection

The plasmid pcDNA3.3p6 was transfected into 293 cells using lipofectamine (Life Technologies). Two to ten μg DNA were complexed to liposomes according to the manufacturer's instructions and transferred to a tissue cell culture dish. Transfections were performed in tissue culture dishes (60mm) or multi-well plates. The amount of DNA as well as the number of cells seeded are illustrated in table 2.5. Cells were prepared one or two days prior to transfection and incubated at 37°C until they reached 60-80% confluence. Supernatant was removed and cells washed twice with 1ml PBS. Subsequently cells were covered with fresh serum free DMEM and incubated for 2h at 37°C. In the meantime the transfection mixture A and B were prepared. Mix A contained the diluted lipofectamine (e.g. for a 6 well dish: 6μl in 94 μl serum free DMEM) and mix B the plasmid, also diluted in serum free DMEM. Prior to transfection both solutions were mixed and left for 15-30min at room temperature to allow complex formation. The transfection mixture was then added

dropwise to the cell monolayer and plates returned to 37°C. The following morning cell supernatant was removed and freshly prepared complete growth medium added. Forty eight to seventy two hours post-transfection supernatant was collected, transfected cells washed twice with PBS and prepared for further applications according to the appropriate protocol.

Dishes	**Number of 293 cells**	**Amount of lipofectamine/final volume**	**Amount of DNA/final volume**
60mm dish	$8x10^5$ per well	10µl per dish/250µl	8µg per dish/250µl
6well dish	$3x10^5$ per well	6µl per well/100µl	5µg per well/100µl
12well dish	$2x10^5$ per well	3µl per well/50µl	2µg per well/50µl

Table 2.5: Number of cells seeded, amount of plasmid DNA and transfection reagent used for transfection of mammalian cells using Lipofectamine.

2.2.6.3b Effectene™ Transfection

Effectene transfection is based on a DNA lipid complex formation. DNA is condensed by a so called 'enhancer' and subsequently the *Effectene* reagent coats this complex with cationic lipids.

The transfection method was optimized following the guidelines provided in the manufacturer's instructions. The optimal ratio of DNA to transfection reagent was determined for each cell line as well as for each plasmid and culture vessel.

In general, a 'mastermix', containing diluted DNA and enhancer was prepared and incubated for 5 minutes at room temperature. Aliquots were then transferred to 1.5ml eppendorf tubes or plastic bijoux already containing the *Effectene* transfection reagent. Tubes were briefly vortexed and incubated for an additional 10min. While complex formation was taking place, cells were washed twice in PBS and overlaid with fresh culture medium. Finally, the DNA complex was diluted in culture medium and applied dropwise to the cells.

Cells were returned to the incubator and harvested 48 to 72 h post transfection. However, if cytotoxicity was observed, the transfection complex was removed, the cell monolayer washed once with PBS and fresh media added.

2.2.6.3c Sodium butyrate induction of transiently transfected cells

To increase recombinant protein expression n-butyrate was added to transfected cells 24h post transfection. Culture medium was removed and replaced by fresh medium containing 2-10mM sodium butyrate. Following another 24h incubation, cells were harvested for recombinant protein detection.

2.2.6.4 Stable Protein Expression

For the establishment of a cell line expressing viral glycoproteins constitutively, plasmids were transfected as detailed for transient transfection. Two days post transfection cells were split in a 1:10 ratio and transferred to a T25 culture flask containing selective media. Media was subsequently changed every five days. After

one to two weeks colonies could be observed. Further cultivation of those selected colonies was performed as described for maintaining mammalian cells. As soon as a sufficient number of cells were available, cells were divided into two T25 tissue culture flasks, grown until they reached 80-90% confluence and subsequently cloned using a 96well dish as well as being stored as nitrogen stocks.

2.2.6.5 Single cell cloning

A confluent T25 flask was harvested using 1ml of trypsin. To achieve a single cell in suspension the flask was shaken vigorously for 30 seconds. Cells were then transferred to a sterile bijoux and counted using a KOVA glasstic slide. One hundred microliters of trypsinised cells were mixed with an equal volume of trypan blue and 10µl were transferred into a chamber of the slide. The number of viable cells in 10 small grids was determined. To obtain cells per microliter the average number of cells per small grid was multiplied by 180.

To obtain a single cell per well, a dilution series was prepared, starting with 100 cells diluted in 10ml selective media. Five millilitres of this cell suspension were transferred to 48 wells, adding approximately 200µl per well by using a 5ml pipette. Subsequently another 100 cells were added to the remaining 5ml of the cell suspension and 2.5ml transferred to 24 wells. Finally, a further 100 cells were mixed with the 2.5ml left in the Universal tube and added to the remaining empty wells.

The plates were incubated until single cells started to divide and colonies had formed. The culture medium was removed and cells were trypsinised as described before. Detached cells were then expanded and tested for recombinant protein expression by various assays.

2.2.6.6 Sodium butyrate induction of stable cell lines

Selected 293 or COS-7 cells were seeded in 60mm^2 dishes or cover slips. Cells were grown until they reached 60-70% confluence. Culture media was removed and cells washed once with 500µl (cover slip) to 2ml (dish) of PBS. Subsequently fresh media supplemented with 2-10mM n-butyrate was added and cells incubated over night at 37°C. The following morning cells were tested for recombinant protein expression by various assays.

2.2.6.7 Detection of cloned insert within mammalian cells by using the Polymerase Chain Reaction

Genomic DNA was isolated using a Genomic DNA Isolation Kit (Promega). Approximately 5x10^6 mammalian cells were harvested and transferred to a 1.5ml eppendorf tube. Following centrifugation for 20sec at 16000 x g cells were lysed in 600µl Nuclei Lysis Solution (supplied by the manufacturer). Subsequently 3µl of RNase solution was added to the nuclear lysate and mixed by inverting the tube 25 times. Further steps, including protein precipitation and DNA precipitation using

Isopropanol were performed as detailed in the manufacturers manual. The pellet, containing the total genomic DNA was resuspended in 50 µl of nuclease free water, supplied by the manufacturer. The extracted DNA was subjected to a standard PCR, amplifying a subfragment of the insert of interest. Oligonucleotide primers and PCR conditions are identical with the previously described colony screen PCR (see above).

2.2.6.8 HepG2 infection

Prior to inoculation supernatant derived from BCP-1 and SY cells was collected in two separate 50ml falcon tube and cleared by 30min centrifugation at 5000rpm. The infectious supernatant was transferred to a fresh 50ml falcon tube and stored at 4°C. Aliquots were collected in 1.5ml screw top eppendorf tubes and stored for PCR testing.

A 90% confluent HepG2 culture was split in 2ml trypsin as described before and 500µl aliquots transferred to four T25 culture vessels. Cells were incubated for 2 days at 37°C (60% confluence). HepG2 cells were washed twice in 1ml PBS and aliquots of supernatant stored at 4°C for PCR testing. The cell monolayer was then overlaid with pre-cleared infectious supernatant either neat or diluted in fresh complete RPMI (dilutions are detailed in the results section). Immediately after inoculation, aliquots of the cell supernatant were removed from each flask and stored for PCR. The following day further aliquots were collected.

Five days post infection supernatant was removed, cell monolayers washed three times with 2.5ml PBS and cells covered in 1ml trypsin. After a 5min incubation at 37°C cells detached and 500µl were transferred to a new T25 culture vessel. Aliquots of supernatant, third wash and detached cells were harvested and stored for further testing. Supernatant and third wash were also centrifuged for 5min at 5000rpm to remove any cells. Two hundred microliter aliquots of both supernatant and wash were then transferred to 1.5 ml eppendorfs. In cases, where a considerable number of detached cells were present in the supernatant the pellet was resuspended in 200µl of PBS and analysed by PCR.

HepG2 cells were split every week and samples taken as detailed above. As this experiment was performed in a category 3 laboratory, the samples were boiled for 5min and tubes wiped down with ethanol prior to transfer.

DNA from cell culture samples was extracted using the Qiagen Mini Blood Kit and tested by nested PCR as detailed above.

2.2.6.9 Sodium butyrate induction of HepG2 cells

It has been shown that sodium butyrate can induce lytic cycle replication of KSHV in PEL cells (Miller *et al.* 1996). HepG2 cells containing KSHV DNA, as indicated by PCR, were split as described above. One culture flask was then incubated with complete RPMI, supplemented with 3mM n-butyrate, whereas the second flask only contained complete RPMI. Two hundred microliters of culture supernatant were

collected 24h after induction. Following another 24h incubation aliquots of culture supernatant, third wash and HepG2 cells were collected and analysed as described earlier.

2.2.7 Detection of recombinant protein expression

2.2.7.1 Preparation of a cell lysate

Transiently transfected cells as well as stably selected cells were lysed prior to recombinant protein detection using several different lysis buffers, described in the literature (Westra *et al.* 1997; Pulford *et al.* 1995).

Supernatant was collected and stored for testing at -20°C. Cell monolayers were washed twice with 1ml ice-cold PBS and detached in 1ml of PBS/EDTA (see Appendix I). The detached cells were transferred to a 1.5ml eppendorf and pelleted at 5000rpm for 5 min. Supernatant was discarded and the cells lysed in 50-100µl of Triton-X-100 lysis buffer (see Appendix I). The lysis was performed on ice for 30min and the extracts subsequently centrifuged for 5 min at14000rpm. The soluble fraction was transferred to a fresh 1.5ml eppendorf tube and the pellet (insoluble fraction) dissolved in 50µl SDS-lysis buffer (see Appendix I). Insoluble protein fractions were sonicated three times for 10 sec at 30 Watts on ice using an ultrasonic probe.

Both protein fractions were stored at -20°C for further applications.

2.2.7.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To detect recombinant protein expression cellular proteins were separated by standard SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a MiniProtean II system (Bio Rad).

Depending on the size of the protein either 7% or 12% polyacrylamide gels were prepared on the day of use (see table 2.6). Prior to loading, samples were mixed with an equal volume of Loading buffer (see Appendix I), and 20 to 30 µl of cell supernatant or cell lysate loaded per well. Protein gels were run at 120 V, 30 mA for 1h30 in 1xPage buffer (see Appendix I) and electroblotted onto PVDF membrane (Amersham) for further detection assays.

To estimate the molecular weight a Rainbow molecular weight marker (Amersham) was used routinely.

	Stacking gel	**Separation gel 7%**	**Separation gel 12%**
30%Acrylamide/2%Bis Acrylamide	500µl	3ml	4.4ml
1M Tris-Cl pH	600µl	4.5ml	4.1ml
10% SDS	25µl	90µl	100µl
Water	3.5ml	4.3ml	2.2ml
10% APS	80µl	60µl	100µl
TEMED	5µl	12µl	5µl

Table 2.6: Composition of the denaturing polyacylamide gels used for protein analyses.

2.2.7.3 Enzyme linked immunosorbent assays (EIA)

For the detection of recombinant glycoprotein expression indirect enzyme linked immunosorbent assays were performed, coating the cell lysate onto plates via *Galanthus nivalis* (GNA) lectin (Roche). Assays were performed in 96 well Nunc Maxisorp plates and 100µl per well were used throughout the assay.

Plates were coated over night at 4°C with 1µg GNA lectin per well in 0.1M $NaHCO_2$. The following morning plates were washed three times with 1xTris-Saline (TS, see Appendix I) and non-specific binding blocked by coating the plates with Blocking buffer (see Appendix I) for 2h at room temperature. Following three further wash steps undiluted cellular lysate or cell supernatant were added to the well and incubated for 2h at room temperature. Wells were washed three times with 1xTS and bound antigen detected using monoclonal antibodies (mAbs) specific for this C-terminal protein tags. The mAbs were diluted in TBS buffer (see Appendix I) supplemented with 3% BSA. To further block cross reactivity, lysed 293 cells were added to the mAbs/TBS mixture and incubated at room temperature for 10 min prior to reaction in the well. Following an 1h incubation at room temperature the washing procedure was repeated and a secondary anti-species goat polyclonal serum, conjugated to horseradish peroxidase and diluted 1 in 1000 in TMTGS (see Appendix I), added. After another 1h incubation plates were finally washed five times with 1xTS, soaking the wells for one minute between each wash. Bound HRP-conjugated antiserum was reacted with TMB substrate to give a coloured product (Murex). TMB substrate and substrate diluent were mixed at equal volumes according to the

manufacturer's directions. One hundred microliters were added per well and the plate incubated for 20-30min at 37°C. Following the observation of blue coloured wells, 100µl of 0.5M HCl was used to stop the oxidisation reaction. The absorbency of each well at 450nm was then determined using a TitreTek MCC multiskan MP (???). Supernatant and cell lysate from mock transfected cells were used as negative controls in all assays.

2.2.7.4 Western Blot

A western blot comprises SDS-polyacrylamide gel electrophoresis, transfer of the proteins to a membrane, antibody incubation and finally the demonstration of the presence of an antibody-protein complex using an antibody probe. In the following procedure different primary antibodies were used. Glycoprotein H, encoding a 6xhistidine tag was incubated with monoclonal antibodies specific for either four or five histidine residues (Qiagen), the monoclonal antibody 9E10, raised against the *c-myc* motif, was used to detect recombinant glycoprotein L. Visualization was performed using the ECL Western Blot System which is based on chemiluminescence (Amersham).

Cell supernatant and protein samples from total cell lysates were resolved by SDS-gel electrophoresis and electrotransferred to PVDF membrane (Amersham) by use of a semi dry blotting apparatus.

Whatman filters were rinsed in the Towbin transfer buffer (see Appendix I) for 15-20 minutes and arranged as illustrated in figure 2.1. Blotting conditions were 10-20 V, 220 mA for 45min and resulted in the transfer of most proteins. To transfer larger proteins the transfer sandwich was left overnight at 60 mA. Prior to protein detection the membrane was pre-wetted with methanol and rinsed with distilled water.

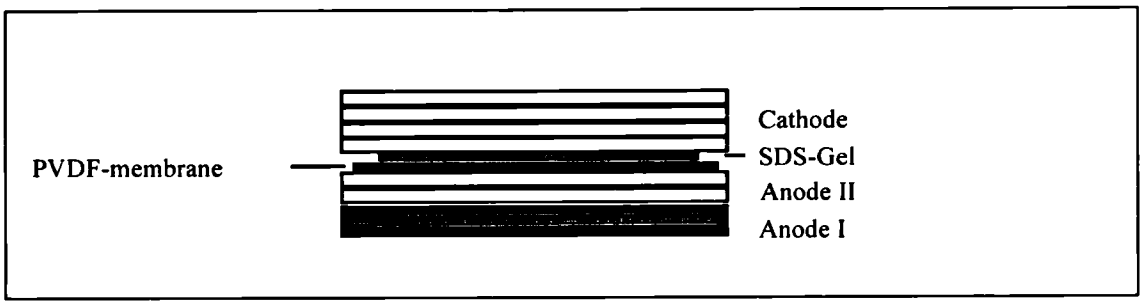


Figure 2.1: Arrangement of SDS-PAGE transfer blot

The membrane was transferred to a sandwich box containing 100ml Tris-Marvel (see Appendix I) and blocked for 90 minutes at room temperature on an orbital shaker. When probing the membrane with anti-his monoclonal antibody, the membrane was blocked in 1% casein instead of milk powder. After being washed three times for 10 min in TBSTT (see Appendix I) the membrane was incubated with primary antibody, diluted in Tris-BSA (9E10) or in Tris-casein (anti-His) (see Appendix I) for 2-3h at room temperature. The anti-His monoclonal antibody was diluted 1 in 2000, 9E10

was used at a 1 in 700 dilution. After three further washes, the anti-mouse HRP conjugated antibody (Harlan Sero Labs), diluted according to the supplier's manual (1:20000) in Tris-NGS (see Appendix I), was incubated with the membrane for 1h. The membrane was then extensively washed (four times for 10min each time) and developed with the ECL Plus Kit. Hyperfilm ECL film sheets (Amersham) were developed using an automated developer.

2.2.7.5 Blue Cell Assay (Clapham et al. 1992)

The blue cell assay is an *in situ* indirect enzyme linked detection assay. Instead of capturing the soluble antigen in a solid phase EIA, transfected cells are tested *in situ* for antigen expression. The primary antibody is specific for the recombinant protein tag and is detected by a species-specific antibody which is β-galactosidase conjugated. The assay is used to assess the frequency of recombinant protein expression after transient transfections as well as to assay stably transfected cell lines. Transfections were performed in 12 or 24 well dishes. Cell supernatant was removed and cell monolayers washed twice with 1ml PBS. Ice-cold methanol acetone was added until cells were covered and dishes left for 30min at -20°C. Following three washes using DMEM supplemented with 2% FCS, recombinant antigen expression was detected using anti-myc monoclonal antibodies 1 in 100 diluted in washing buffer. Dishes were placed on a slow orbital shaker and incubated for 2h at room temperature. Subsequently cells were washed three times and incubated with the

second antibody, a goat anti-mouse β-galactosidase conjugate (diluted 1:600) (Harlan Sero Labs), for 1h. Washing steps were repeated and cells were finally washed twice with PBS to remove any residual DMEM. The substrate X-Gal (Roche Diagnostics) was diluted 1 in 100 (f.c.0.5mg/ml) in staining solution (see Appendix I). Blue coloured cells were visible after two hours. To preserve the colour reaction, plates were incubated in the dark.

2.2.7.6 Indirect immunofluorescence confocal microscopy

The intracellular localisation of the recombinant proteins was analysed by indirect immunofluorescence using a Bio Rad MRC1024 Confocal System. Images were overlaid using NIH Image 1.61 software.

Cells were either directly transfected in coverslips or seeded onto coverslips (Life technologies) after the selection process.

2.2.7.6a Staining of fixed cells

The culture media was removed and cells washed twice with 1ml of ice cold PBS. Subsequently cells were covered with ice-cold methanol/acetone and left at -20°C for 30min. After three washes with DMEM, supplemented with 2%FCS, non-specific binding sites were blocked in DMEM containing 5% normal goat serum (NGS) for 30min at room temperature. The wash steps were repeated and the antibody, diluted 1 in 20 in washing buffer, added. Following a 1 hour incubation at 37°C cells were

washed again and further incubated with a goat α-mouse FITC conjugated antibody (1:20 diluted) (Dako) for 60 min. The dilution buffer was supplemented with 2% normal goat serum. Finally cells were washed thoroughly 3 times with washing buffer and 2 times with PBS. Slides were air dried and covered with a glass coverslip using mounting fluid (Dako). All incubation steps were performed on an orbital shaker. For counterstaining of DNA cells were mounted using VECTASHIELD mounting medium containing 1.5μg/ml propidium iodide (PI) (Vector Labs). In addition to methanol/acetone fixation some cells were incubated for 15min with 2% paraformaldehyde in PBS. After three wash steps, cells were permeabilized for another 15min in 0.1% Triton-X-100 in PBS or in acetone for 5 minutes. Subsequent staining was performed as detailed above.

2.2.7.6b Staining of viable cells

To detect possible surface expression cells were stained on ice prior to fixation. Cells were harvested and labelled as described above. Following the last wash step cells were fixed in methanol/acetone and prepared for confocal microscopy as detailed in section 2.2.7.6a.

2.2.7.6c Staining of Golgi organelles

For experiments in which cells were double labelled for recombinant glycoprotein L and for Golgi organelles, BODIPY TR ceramide (Molecular Probes), a golgi marker,

was added at a final concentration of 0.5 to 1μM during incubation with secondary antibody. BODIPY TR ceramide has spectral properties similar to those of Texas Red.

2.2.7.7 Flow-cytometric analysis

Stable cell lines and transiently transfected cells were analysed by Flow cytometry (FACS) for recombinant protein expression.

Harvesting

For transient transfections mammalian cells were transfected in 60mm dishes as described above. At 48h post transfection, tissue culture supernatant was removed and the cells washed twice with 2ml PBS. Cells were then harvested using 1ml of trypsin and transferred to 3 ml polystyrene round bottom tubes (Falcon 1260). Viable cell counts were determined (trypan blue exclusion) and cells pelleted (5min at 2000rpm). Cells were resuspended in PFA (see Appendix I) at 10^6 cells per ml.

Stably transfected cells were harvested using 1ml of trypsin and transferred to falcon tubes as described above.

Staining

A total of 10^5cells were incubated with 100µl of primary antibody (mAbs diluted 1 in 50 in PFA) for 1h at room temperature. To fix cells, 40µl of PermaFix (ORTHO Diagnostics) was added to each tube.

Cells were washed by adding 5ml of PFA followed by centrifugation at 2000rpm for 5min. Cells were resuspended in 100µl of PFA, supplemented with 2% normal goat serum, containing goat anti-mouse FITC conjugated antibody (Dako) at a 1 in 30 dilution. After incubation for 1h at room temperature, the cells were washed twice with 5 ml PFA and finally resuspended in 100µl of PFA. 10000 cells were analysed using a FACScan apparatus (Becton Dickinson). Data obtained was analysed with the software Win MDI Version 2.8.

3. Expression of the KSHV glycoprotein H and glycoprotein L

3.1 Introduction

KSHV is implicated in the pathogenesis of Kaposi's sarcoma (KS). The viral genome has also been identified in other lymphoproliferative diseases such as primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). Herpesviruses encode a large number of glycoproteins which are involved in viral attachment and viral penetration into the host cell. The herpesviral glycoprotein H is highly conserved throughout the herpesvirus family and studies demonstrate its importance in viral fusion processes. Recombinant expression of authentic herpesviral glycoprotein H and its transport to the cell surface has been demonstrated to depend on the presence of a second viral glycoprotein, glycoprotein L. Sequence homology searches have identified gH and gL homologs within the genome of KSHV. Using nested PCR KSHV ORF 22 (gH) and ORF 47 (gL) were amplified and cloned into the mammalian expression vector pcDNA3.3p6. Oligonucleotide primers were designed to encode different C terminal protein tags for each protein to facilitate detection when co expressed. The generated clones, named pcDNA3.3p6.22, and pcDNA3.3p6.47 were co- or separately transfected into HEK293 cells and subsequent protein expression analyzed. In addition, successfully transfected cells were selected and tested for protein expression.

3.2 Expression systems for the production of viral glycoproteins

Several protein expression systems have been developed, all having their advantages and disadvantages. To study the function of viral glycoproteins their glycosylation pattern has to be as authentic as possible to maintain confirmation and possible epitopes that are important in immunological recognition. The synthetic production of eukaryotic proteins frequently requires post-translational phosphorylation, oligomerization, or specific proteolytic cleavage processes that are not performed by bacterial cells. As demonstrated for the expression of the HIV-1 gp120 glycoproteins *E. coli* and yeast produce a recombinant protein that is neither glycosylated nor correctly folded (Sohn *et al.* 1996). To synthesize recombinant glycoprotein that most closely reflects the wild type glycosylation pattern of the protein, expression systems based on mammalian cells are recommended. However, some cell lines are more productive than others and the choice of an expression vector is crucial in optimizing expression levels.

3.2.1 Choice of mammalian cell line

Several mammalian cell lines are widely used for expression studies, including CHO, Cos7 and HEK293 cells. However, each cell line shows a different glycosylation pattern (Mizuochi *et al.* 1990). As mentioned before, viral glycoproteins play an important role in immune response of the host cell and antibody recognition may depend on oligosaccharide structures (McKeating *et al.* 1992). Thus a mammalian expression system should be selected which allows optimal glycosylation as well as a high yield of recombinant protein. The human embryonic kidney cell line (293HEK) has been extensively used in transient and

stable protein expression for a variety of proteins (Fao-Tomasi *et al,* 1991; Brightwell *et al.* 1997). The cell line is adherent and shows a high transfection efficiency.

3.2.2 Choice of mammalian expression vector

Several aspects have to be considered when selecting an expression vector. For the establishment of a stable cell line the vector has to encode a selectable marker which confers resistance to an antibiotic or other drug. Secondly, the promoter should provide a strong 'drive' for protein expression. The vector pcDNA3.3p6 has been designed and generated in the Department of Virology, UCL (J. May, PhD thesis, 1998) and contains the neomycin gene, enabling transfected cells to grow in the presence of geneticin to select positive transfectants. Furthermore, the original hCMV promoter has been replaced by the major immediate-early (MIE) gene enhancer-promoter of human cytomegalovirus (hCMV) that contains the 5' untranslated sequence (Intron) of the MIE gene (Accession number M21295; Akrigg *et al.* 1985). Studies have showed that the additional intron increases expression levels in stable as well as transient transfections (Boshart *et al.* 1985; Stephens and Cockett, 1989)

However, newly synthesized proteins must also be targeted to their functional compartment within the cell. A signal sequence located at the N terminus of the protein translocates the pre-protein into or across the endoplasmatic reticulum and Golgi for post translational modifications (Wilkinson *et al.* 1997). Signal sequences contain a hydrophobic core but show great variation both in overall

length and amino acid sequence. Some signal sequences are less efficient than others and the yield of expressed protein is decreased (Li *et al.* 1994). The replacement of the natural signal sequence by an artificial sequence has been shown to increase the level of protein produced (Li *et al.* 1994). In particular the signal sequence from the tissue plasminogen activator (tPA) has been used successfully (Golden *et al.* 1998; Rhodes *et al.* 1994). The vector, pcDNA3.3p6, used for the expression of gH and gL encodes the tPA leader sequence (between HindIII/BamHI restrictions sites of the polylinker) upstream of the inserted open reading frame. The design and generation of this vector are described in detail in the PhD thesis of J. May (1998). A plasmid map is given in Appendix II.

3.2.3 Cloning of KSHV ORF22/gH and ORF47/gL

Prior to amplification of the two viral open reading frames, their amino acid sequences were analysed for the presence of a signal sequence and possible transmembrane domains. The sequences predicted to encode gH and gL were obtained from GenBank (Accession number: U75698). The region spanning nucleotide 37113 to 39305 (ORF22) is predicted to encode gH, and nucleotides 69412 to 69915 (ORF47) are considered to encode the gL homolog. Following prediction of the signal sequences oligonucleotide primers were designed and the PCR conditions optimized. Once amplicons were obtained the DNA fragments were cloned into the mammalian expression vector pcDNA3.3p6.

3.2.3.1 Computer-assisted characterization of KSHV ORF22/gH and ORF47/gL

Computer analysis of the amino acid sequences corresponding to KSHV ORF22/gH revealed characteristics of a membrane spanning glycoprotein; including an N terminal hydrophobic signal, a transmembrane sequence, a putative cytoplasmic tail and the presence of N-linked glycosylation sites (Schlesinger and Schlesinger, 1987). In figure 3.1 a hydrophilicity plot of the KSHV ORF22/gH along with the predicted glycosylation sites, indicates two regions that may be membrane transversing. If the average hydrophobicity of a region of 19 amino acids is greater than 1.6, a high membrane spanning probability may be predicted (Kyte and Doolittle, 1982). In fact, ORF22/gH encodes two such regions, one at the N terminus and one towards the carboxy terminus (Fig 3.1A, underlined). The first 17 amino acid residues at the N-terminus could serve as a signal sequence for co-translational translocation of the protein across the endoplasmatic reticulum. This sequence is similar in length and composition to other known eukaryotic signal sequences (McGeoch, 1985; von Heijne, 1986) A signal cleavage site may be predicted after amino acid 17, using evaluations of amino acid counts in each position of known eukaryotic signal sequences. Cleavage at this site would result in a protein of 713 residues with a predicted molecular weight (M_r) of 79kDa. The protein sequence also contains 15 potential N-glycosylation sites (as indicated by peaks). Furthermore, the highly conserved glycosylation site NGTV could be identified at position 688 to 691 (sequence not shown). This motif for N-linked glycosylation has been described for all glycoprotein H homologs.

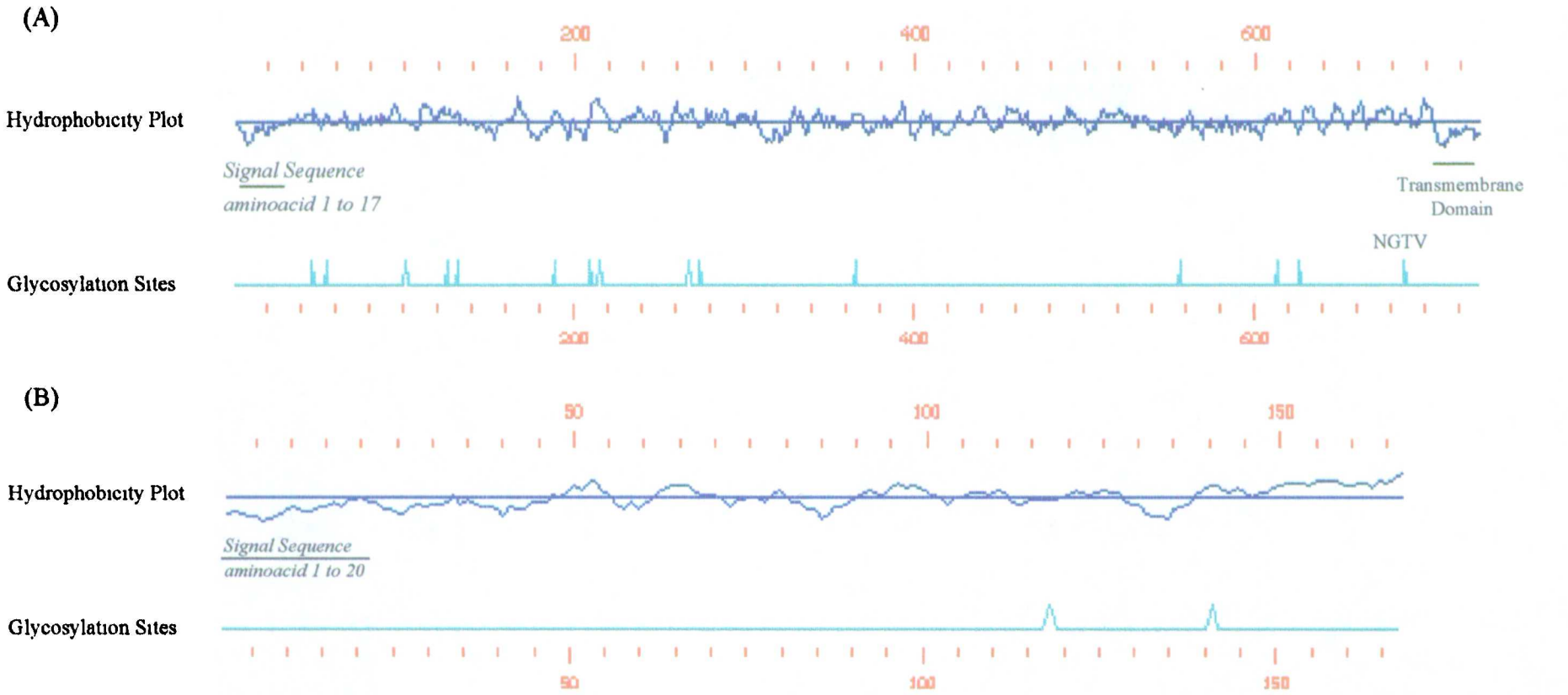


Figure 3.1: Kyte-Doolittle hydropathy analysis of (A) KSHV ORF22/gH and (B) KSHV ORF47/gL. Areas above and below the line denote hydrophilic and hydrophobic domains, respectively. The scale above the line graph indicates the amino acid residues. Predicted signal sequences are underlined in black, possible transmembrane domain is indicated. Evaluations were determined using the PREDICT program of the G-C-G package.

Analysis of ORF47/gL, presumed to encode the homolog to the herpesviral glycoprotein L, also showed evidence of a hydrophobic domain at the amino terminus, suggesting that this region also encodes a signal sequence (figure 3.1B). The cleavage site is most likely after amino acid 20, leaving a 147 amino acid polypeptide of M_r 16kDa, containing 2 potential N-glycosylation sites. As indicated in the hydrophilicity plot, no evidence for a membrane spanning region could be detected, despite the theoretical signal sequence.

3.2.3.2 Choice of DNA samples

KSHV has been identified in KS, primary effusion lymphoma and multicentric Castleman's disease. The overall variability between different viral isolates is suggested to be low, approximately 1-1.5% (Zong *et al.* 1997). Glycoprotein H is highly conserved throughout the herpesvirus family. A recent study examining KSHV transmission between individuals from different geographic regions confirmed that KSHV gH also shows low sequence variability, less than 0.6% respectively (Meng *et al.* 1999). Therefore, only two different isolates were selected for initial expression studies. Total DNA was purified from BCP-1 supernatant, representing cell culture adapted virus, and from blood of a classic KS patient. Sequences derived from BCP-1 supernatant are referred to as ORF22S for gH and ORF47S for gL, while ORF22C and ORF47C indicate the 'classic' KS origin.

3.2.3.3 Amplification of KSHV ORF22/gH and ORF47/gL

The number of viral DNA molecules in each sample was assessed using limiting dilution PCR (see section 2.2.2.1). For the amplification approximately 10^3 DNA molecules were added to a 50µl PCR reaction mixture. The amplification of KSHV ORF22/gH was optimized by testing a range of annealing temperatures and primer concentrations. An optimal result was achieved by changing both parameters (see section 2.2.2.2a for a detailed description). The combination of a 54°C as annealing temperature and a higher concentration of primers resulted in the amplification of an approximately 2100bp DNA fragment as illustrated in figure 3.2A. The polymerase chain reaction amplifying glycoprotein L was performed using the reaction conditions as described for gH. The separated 480bp DNA fragments are shown in figure 3.2B. The oligonucleotide primers used in these reactions are described in chapter 2.

3.2.3.4 Cloning of the desired fragments

The amplified DNA fragments, encoding KSHV ORF22/gH and ORF47/gL were recovered from agarose gel electrophoresis and cloned into pcDNA3.3p6 using the Clone Amp reaction originally described by Life Technologies (May J., PhD thesis, 1998). Following transformation into *E. coli* SCS-1, plasmids were purified and digested with the restriction enzymes *EcoRI* and *XhoI*. To avoid possible PCR-derived point mutations two to three clones were selected and analysed. As ORF22/gH exhibits restriction sites for these enzymes, positive digests resulted in three fragments with sizes of approximately 130bp (EcoRI/EcoRI), 690bp (EcoRI/XhoI) and 1400bp (XhoI/XhoI) respectively (figure 3.3A, lanes 5-8).

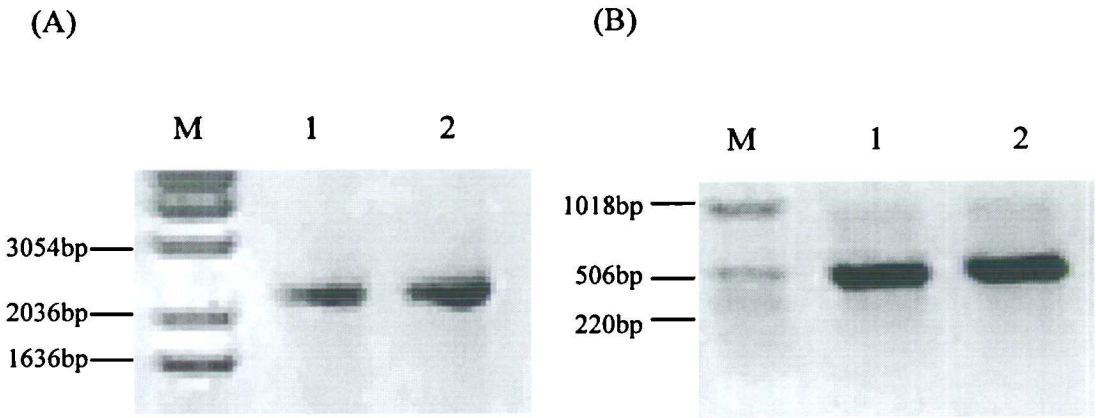


Figure 3.2: Agarose gel (1%) analysis of the PCR amplification products. Total DNA was extracted and 1µl was subjected to nested PCR as detailed in the text.
(A) KSHV ORF22 (gH); M: 1kb DNA ladder (Life Technologies); lane 1: ORF22 from classic KS patient; lane 2: ORF22 from BCP-1 supernatant
(B) KSHV ORF47 (gL) M: 1kb DNA ladder (Life Technologies); lane 1: ORF47 from classic KS patient; lane 2: ORF47 from BCP-1 supernatant
Numbers on the left hand side of each picture indicate the molecular marker.

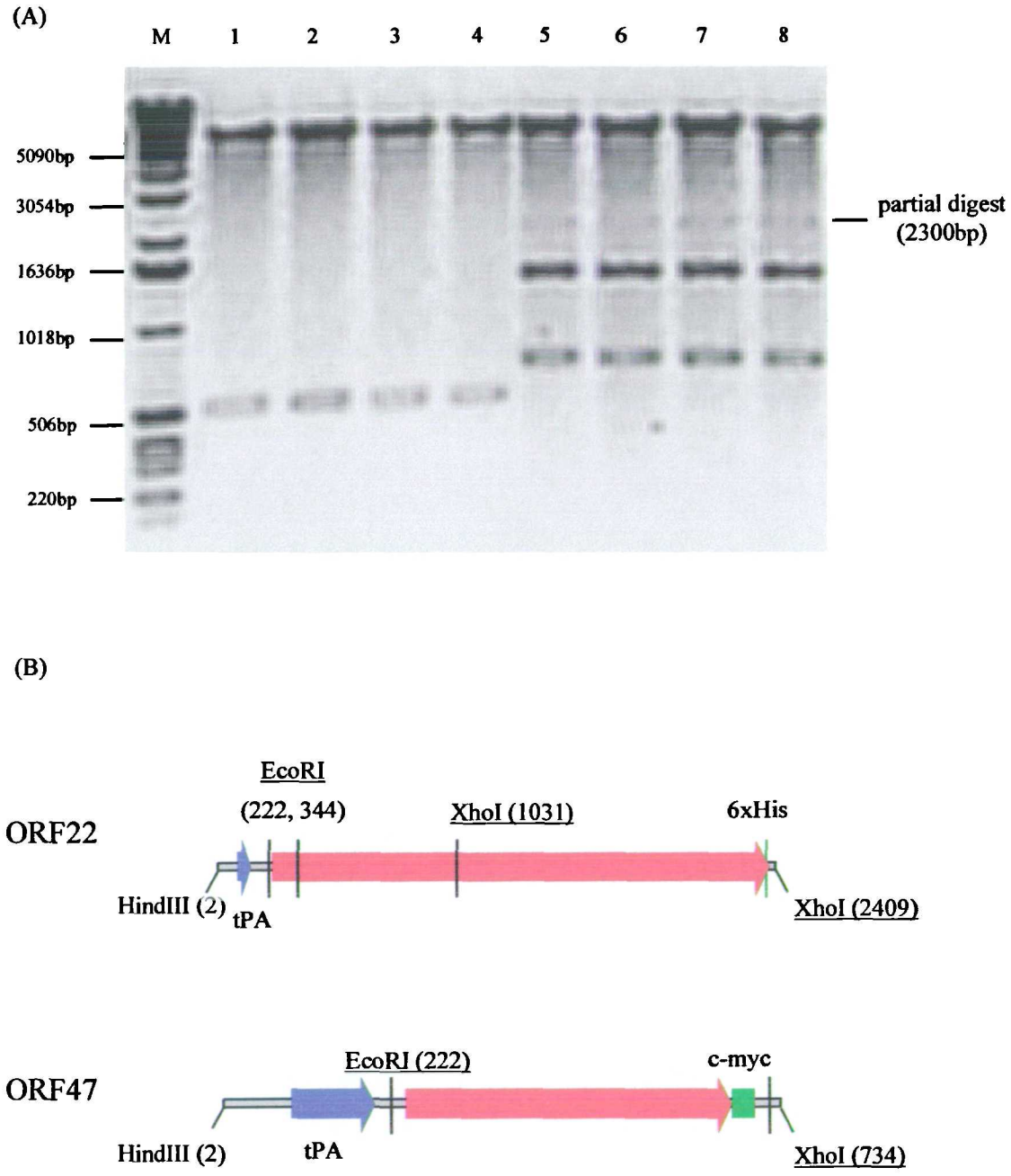


Figure 3.3: (A) Agarose gel analysis (1%) of the DNA fragment pattern after EcoRI/XhoI restriction digest. Plasmids were purified and digested with the restriction enzymes EcoRI and XhoI. Lane 1: pcDNA3.3p6.47S.2; lane 2: clone 8; lane 3: pcDNA3.3p6.47C.9; lane 4: clone 4; lane 5: pcDNA3.3p6.22S.4; lane 6: clone 10; lane 7: pcDNA3.3p6.22C.16; lane 8: clone 20; M: 1kb DNA marker (Life Technologies). (B) Schematic picture of the HindIII/XhoI fragment encoding KSHV ORF22/gH and ORF47/gL that were inserted in pcDNA3.3p6. Blue: tPA leader sequence; red: KSHV ORF, green: protein tags. EcoRI/XhoI cut site coordinates shown in brackets.

However, a faint 2300bp fragment could also be detected, suggesting a possible partial digest (EcoRI-XhoI-XhoI). In contrast, for plasmids encoding ORF47/gL, the enzymes *EcoRI* and *XhoI* release the insert, resulting in a 510bp DNA fragment (lanes 1-4). Positive clones were named either pcDNA3.3p6.22S or pcDNA3.3p6.47S followed by the clone number, for sequences derived from BCP-1 supernatant or pcDNA3.3p6.22C and pcDNA3.3p6.47C for sequences derived from an individual with classic KS. In theory these constructed plasmids should be able to express the KSHV homolog glycoproteins with a) a N terminal tPA leader sequence for efficient post-translational modification and b) a C terminal protein tag to facilitate the detection of recombinant protein expression. For detection of each glycoprotein in co-expression studies glycoprotein H encoded six histidine residues, while glycoprotein L contained the c-myc epitope. A schematic diagram of the HindIII/XhoI DNA fragments encoding gH and gL as cloned into the expression vector is given in figure 3.3B. Numbers indicated the restriction sites within the fragment; number one is defined at the HindIII site.

3.2.4 Transient transfection experiments

Transient transfections allow a rapid analysis of recombinant protein expression. All plasmids were initially tested in transient transfections to establish the level of protein expression. Transfection procedures were optimized using the control plasmid pcDNA3.1-his-β-Gal (see Appendix II for plasmid map), expressing the β-galactosidase with six N terminal histidine residues. Transfection efficiency was scored by counting the number of blue cells detected per well. HEK293 cells were

transfected at 60-70% confluence using lipofectamine (Life Technologies). Two days post-transfection cells were fixed in a 1:1 methanol/acetone solution and the substrate X-Gal added. Following a one hour incubation blue cells were counted by eye. Routinely 20-30% of the total cell population could be transfected (data not shown).

Each plasmid construct was tested in a time course experiment to detect optimal protein expression of ORF22/gH and ORF47/gL. Expression levels were assessed by blue cell assay after 48, 72, and 96 hours post-transfection. Several control transfections were performed to establish the activity of the reagents and antibodies as well as non-specific binding of the monoclonal antibodies 9E10 (ICRF) and penta-His (Qiagen). As a control for transfection efficiency pcDNA3.1-β-galactosidase was used; to monitor nonspecific binding, water was transfected; to define assay conditions, a plasmid (pSEC-GBV-C E2) encoding the histidine and c-myc tagged glycoprotein GBV-C E2 was transfected (kind gift of Dr R. B. Ferns). It was found that the anti-histidine monoclonal antibody failed to detect any protein in the blue cell assay procedure. However, western blot procedures confirmed the activity of this mAb against the histidine control protein (see below). Therefore, the initial detection was focused on cell lines expressing proteins with the c-myc epitope, as the histidine antibody could not be optimized for these *in-situ* assay procedures.

3.2.4.1 Blue cell assay

Plasmid constructs were transfected in 12-well dishes as described in chapter 2.2. Detection of recombinant protein was performed by using monoclonal antibodies specific for the myc epitope (9E10). The primary antibody was tested at different dilutions. Unfortunately, the level of expression for the plasmids encoding glycoprotein L was nearly undetectable (data not shown). In a confluent 31 mm^2 dish only ten blue colored cells could be observed, regardless which plasmid was used and at which time point cells were fixed. In the case of control transformation of non expressing 293 cells the number of stained cells detected was similar, suggesting that the low number detected was due to cross reactivity of the primary antibody. Positive controls using the GBV-C E2 plasmid confirmed the usual transfection efficiency and the activity of the antibody in-situ. The establishment of stable cell lines may favor the possibility of an increase in the number of expressing cells.

3.2.5 Detection of recombinant protein in stably transfected cells

Following transient transfection experiments, successfully transfected HEK293 cells were selected in the presence of neomycin. As soon as a reasonable number of cells was available, cells were expanded and aliquots analyzed by a variety of methods, as well as stored in nitrogen. The names of the selected cell lines are listed in table 3.1.

Cell line	transfected plasmid	inserted sequence
KS22S or KS22C*	pcDNA3.3p6.KS22	ORF22/GH (glycoprotein H)
KS47S or KS47C*	pcDNA3.3p6.KS47	ORF47/GL (glycoprotein L)

*S: sequences derived from BCP-1 supernatant; C: sequences derived from clinical sample

Table 3.1: Stable cell lines containing KSHV ORF22/gH or ORF47/gL. Following transient transfection HEK2 93 cells were selected as detailed in Material and Methods. Cell lines are positive by PCR for the appropriate insert.

3.2.5.1 Verification of the presence of the expression plasmids within HEK293 cells

The presence of the expression plasmids within selected HEK293 cells was verified by polymerase chain reaction. One hundred microliters of total genomic DNA were prepared from approximately 10^5 cells and one microliter subjected to a standard PCR (see section 2.2.4.7). Amplification primers were complementary to a region within the glycoprotein coding sequence, resulting in a 778bp screening fragment for KSHV glycoprotein H and 320bp for glycoprotein L. Agarose gel analysis of the PCR products revealed the presence of the appropriate plasmids, encoding the KSHV glycoproteins, within the respective cell lines (see figure 3.4).

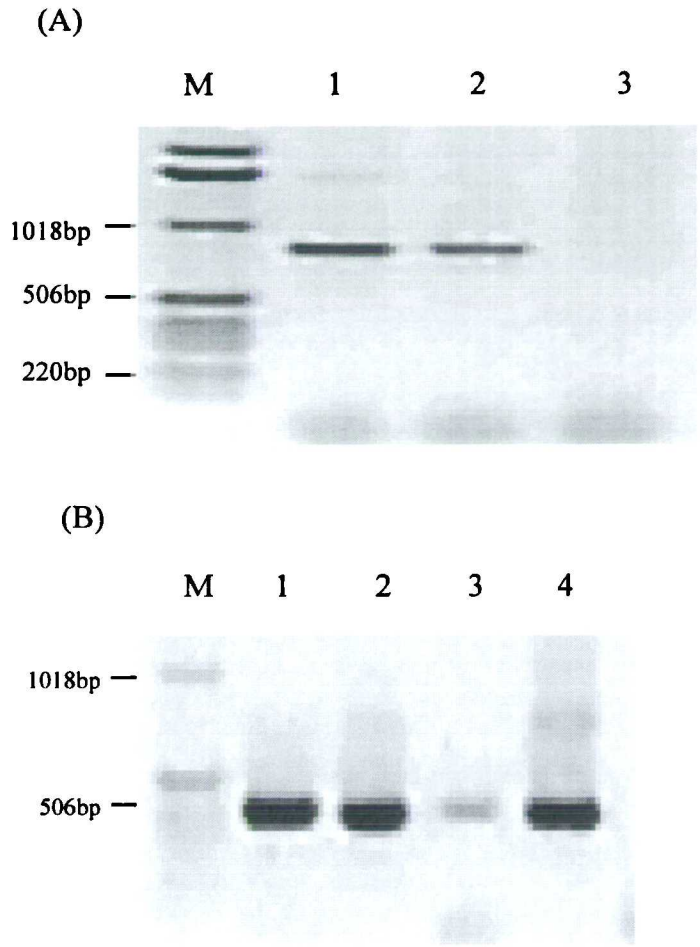


Figure 3.4: Polymerase chain reaction of selected HEK293 stable cell lines. Tota DNA was isolated and one microliter was amplified by PCR as detailed in the text Ten microliters were analysed on a 1% agarose gel.

(A) Analyses of cell lines expressing ORF22/gH. Lane 1: 293KS22S.2; lane 2 293KS22C.3; lane 3: negative control

(B) Analyses of cell lines expressing ORF47/gL. Lane 1: 293KS47S.2; lane 2 293KS47C.5; lane 3: 293KS47C.2; lane 4: pcDNA3.3p6.KS47S plasmid a positive control.

3.2.5.2 Screening of protein expressing cell lines by blue cell assay (BCA) and indirect immunofluorescence

As mentioned above, the monoclonal antibody penta-his failed to detect histidine tagged proteins in blue cell assay procedures. Furthermore, screening for ORF22/gH-his by indirect immunofluorescence was unsuccessful as the antibody could not detect protein in this assay either. A duplicate transfection, was performed using the control plasmid pcDNA3.1-his-β-galactosidase (Invitrogen). The expressed β-galactosidase contains six amino terminal histidine residues and can be detected either in a color reaction using the substrate X-Gal or by using the monoclonal antibody penta-His. Following transfection one well was stained as detailed before and the second well reacted against the antibody. Although the usual transfection efficiency of around 30% was seen in the first well the indirect immunofluorescence failed to detect any recombinant protein, only nonspecific binding being observed. Consequently, testing for the presence of the ORF22/gH gene product had to be based on enzyme linked immunoabsorbent assay (see 3.1.4.3) or western blot. Similarly, detection of ORF47/gL-c-myc by immunofluorescence was unsuccessful. Blue cell assay studies were performed to test the stable cell lines KS47S or KS47C. Selected HEK293 cells containing the KSHV ORF47/gL were seeded at a density of 10^4 cells per well (48 well dish) and grown until they reached 70-80% confluence. Following fixation and permeabilization of the selected cells, monoclonal antibodies specific to the c-myc protein tag failed to detect any protein in the tested cell lines; thus blue coloured cells could not be observed. Positive controls confirmed in-situ reactivity of this

antibody. Background staining could not be detected in non transfected 293 cells (data not shown).

Indirect immunofluorescence studies were also performed on the same gL-transfected stable cell lines and showed no specific staining, confirming the previous results obtained from blue cell assays.

3.2.5.3 Detection of the ORF22/gH gene product using a lectin based EIA

Herpesviral glycoprotein H homologs have all been identified in the cytoplasm when expressed in a variety of systems (Duus and Grose, 1996; Pulford *et al.* 1995; Cranage *et al.* 1988; Gompels and Minson, 1989). Therefore this detection system was optimized for the analysis of cell lysate rather than cell supernatant. However, initial experiments testing cell culture supernatant were negative for protein expression (data not shown). Different formats of indirect enzyme linked immunsorbent assays were used. Cellular lysate or supernatant was captured onto a solid phase either directly by association with lectin, or via the C terminal protein tag (figure 3.5). According to computer analysis, the protein product of KSHV ORF22/gH contains 15 glycosylation sites. This offers the opportunity to capture the glycoprotein via lectin onto a solid phase. Lectins are able to bind specifically to carbohydrate structures in glycoproteins. This property makes them a useful tool in the characterisation of glycosylation patterns. The lectin used in the following EIA derives from *Galanthus nivalis* and exhibits specificity for terminal mannose residues. Coated on a solid phase it captures the recombinant glycoprotein, presenting it for further detection by anti-sera.

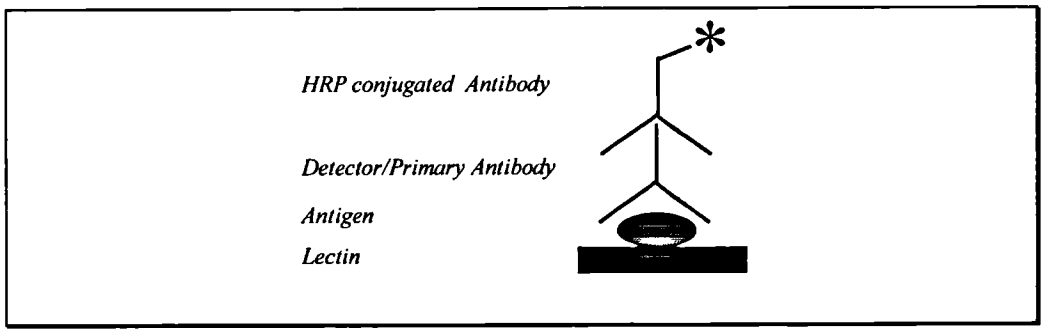


Figure 3.5: Schematic picture of the EIA performed in recombinant protein detection. Antigen represents the HEK293 lysate or supernatant as well as water. Detector or primary antibody is either serum or monoclonal antibody.

Antigens captured via lectin were detected with serum derived from KSHV patients as well as with the anti-histidine monoclonal antibodies. Prior to use in ELISA serum was tested in an indirect immunofluorescence assay using KSHV infected BCP-1 cells. The typical nuclear stippling pattern could be observed, indicating the presence of antibodies against the viral latent nuclear antigen LANA-1 (see figure 3.6, kindly provided by U. Ayliffe). Monoclonal antibodies were tested in western blots (see below).

3.2.5.3a Serum detection

Stable cell lines were grown until they reached 80-90% confluence, approximately 10^6 cells were harvested in 100µl lysis buffer A (see Appendix I). To determine non-specific binding of antibodies, non-transfected 293 cells were treated in the same way. Serum obtained from virus-infected patients showed high background

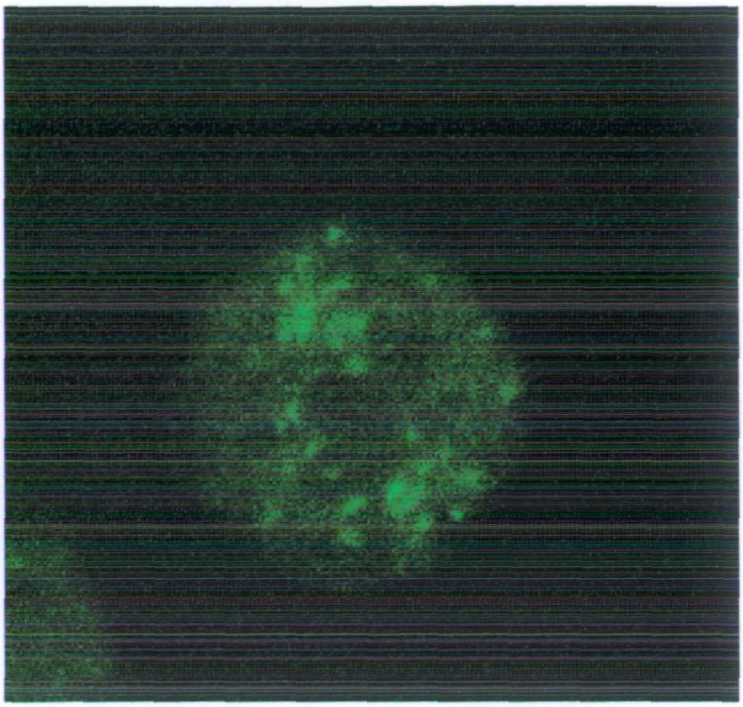


Figure 3.6: Indirect immunofluorescence of BCP-1 cells using serum from KS positive patients. The picture shows the typical stippling pattern, characteristic for antibodies against the viral nuclear antigen (LANA). The picture was kindly provided by U. Ayliffe.

levels when reacted against the negative control cell lysate. Previous experiments revealed that this non-specific binding of the sera could be reduced by the presence of 293 cell lysate during antibody-lysate binding (data not shown). Therefore the primary antibody mixtures were pre-incubated for 10 min with a 293 cellular lysate.

In the indirect EIA using lectin on the solid phase the optical density was compared to serum dilutions, as shown in figure 3.7A and B. Despite the addition of 293 cell lysate to the anti-sera during incubation, KSHV serum showed cross reactivity with non-transfected 293 cell lysate (negative control) as illustrated by black squares. Furthermore, an elevated optical density was observed when water was used as antigen (black triangles).

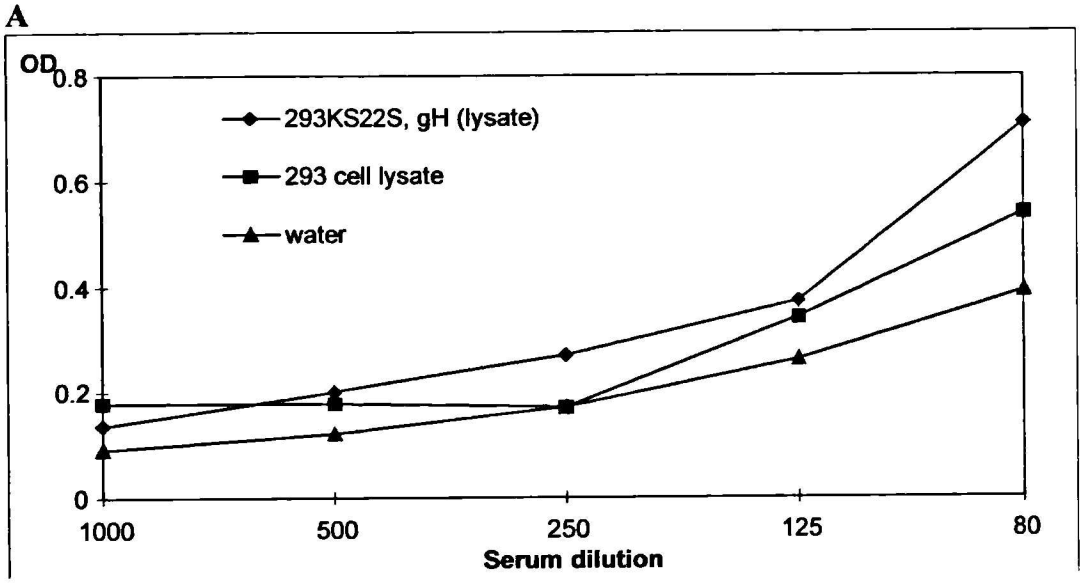
In summary 293 cells containing the expression plasmid (diamond shape) showed an increase in optical density (OD) compared to non-transfected 293 cell lysate, also a serum dilution dependence could be noticed. A two fold dilution series of the patient serum showed a proportional increase between the optical density and the serum dilution. However, the observed difference in optical density between transfected cells and non-transfected cells (293 cell lysate) was small, less than two fold. The difference between negative control and supposedly expressing cell lines was even less when analysing 293KS22C cells, as illustrated in picture 3.7B. The optical density values were almost identical.

Supernatant from transfected cells was tested as described above and similar results obtained (data not shown). None of the cell supernatants showed any expression of recombinant glycoprotein, indeed the OD values obtained by serum

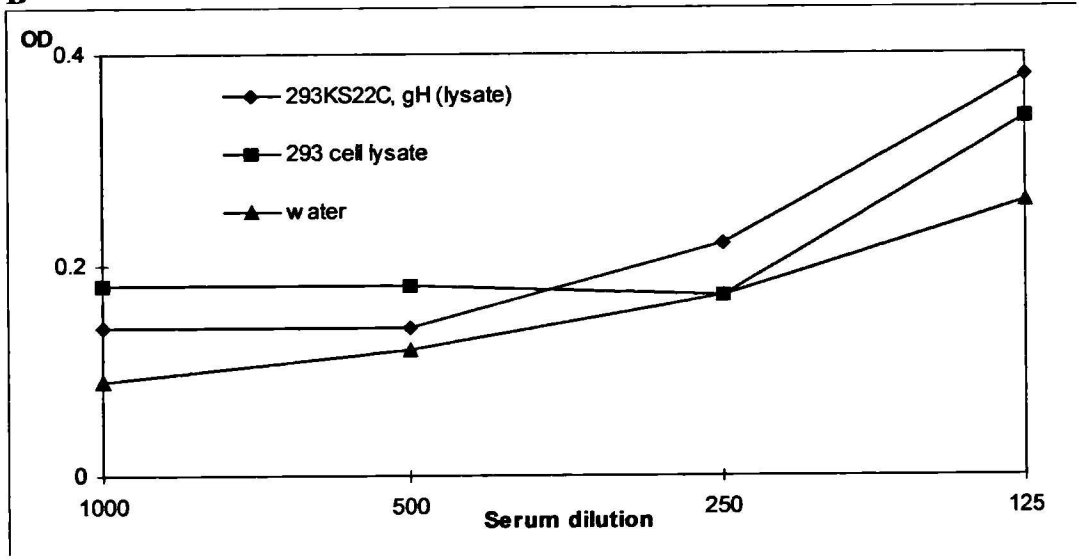
detection were lower than the negative controls. This is unsurprising as herpesviral glycoprotein H has been described as a cytoplasmic protein in recombinant protein expression studies.

Figure 3.7: Detection of recombinant KSHV glycoprotein H in HEK293 cells using an indirect EIA. Cellular lysate was captured via lectin onto a 96 well plate. Antigen was detected by serum from KSHV infected patients (A,B) and using a monoclonal antibody specific for five histidine residues (C). A: 293 cell line transfected with pcDNA3.3p6.KS22S; B: 293 cell line transfected with pcDNA3.3p6KS22C. C: Lysate from different stable cell lines was collected at different time points. Numbers along the Y-axis indicate the optical density at 450nm.

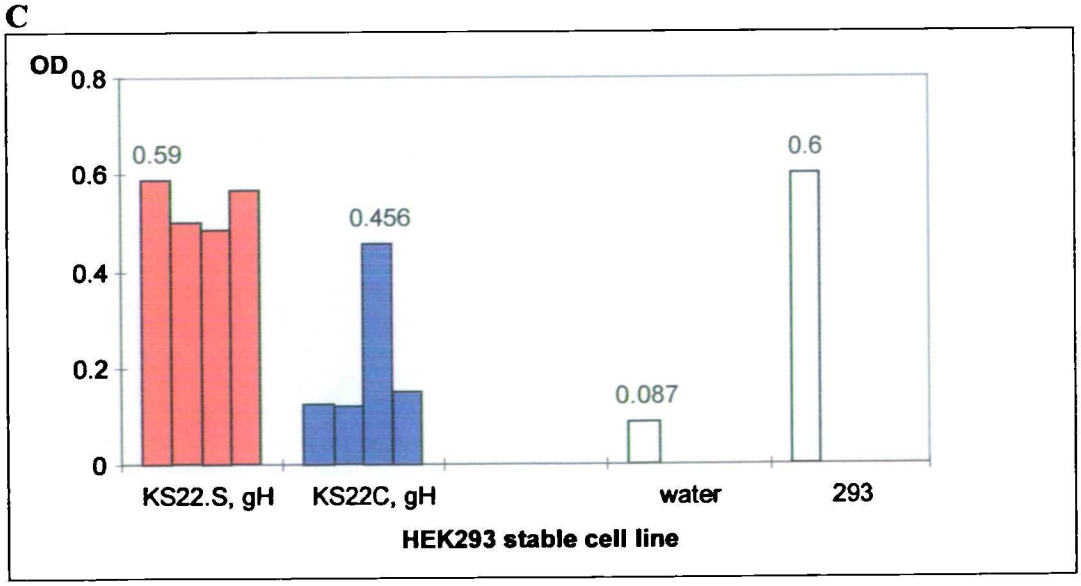
A


OD
0.8
0.6
0.4
0.2
0
293KS22S, gH (lysate)
293 cell lysate
water
1000
500
250
125
80
Serum dilution

B


OD
0.4
0.2
0
293KS22C, gH (lysate)
293 cell lysate
water
1000
500
250
125
Serum dilution

C


OD
0.8
0.6
0.4
0.2
0
0.59
0.456
0.087
0.6
KS22.S, gH
KS22C, gH
water
293
HEK293 stable cell line

3.2.5.3b Protein tag detection

When using an EIA based on monoclonal antibodies specific for the encoded six histidine residues a similar result was observed. Figure 3.7C shows OD values plotted against samples, prepared at different stages during the selection process. Cell lysate was captured via lectin onto a solid phase and tested for the presence of histidine tagged protein. Non-specific binding could be reduced when incubated in the presence of non transfected 293 lysate, but the detection of recombinant protein was inconsistent, as indicated by the red and blue bars. Both elevated and low levels of signal could be detected when analysing the gH stable cell line. However, compared to non-transfected cells (white bars) OD values were lower, suggesting that no recombinant gH has been produced by the selected cells. Similar results were obtained for HEK293 cell lines transfected with plasmids containing KSHV ORF47/gL (data not shown). In conclusion, neither serum nor monoclonal antibody were able to detect recombinant protein.

3.2.5.4 Western Blot

To eliminate the possibility that the complex tertiary structure of the recombinant proteins occlude the protein tags and prevent binding of the antibodies, western blots of cell lysate and supernatant were performed. Initially monoclonal antibodies were tested for reactivity and western blot procedures for each antibody optimized. HEK293 cells were transiently transfected with the control plasmid pcDNA3.1-his-β-galactosidase (see Appendix II for plasmid map). After 48h cells were harvested, lysed in laemmli buffer and $2x10^5$ cells per well separated on a 12% SDS polyacrylamide gel. Proteins were transferred onto PVDF membrane

and reacted with monoclonal antibodies followed by a species-specific HRP-conjugated antisera. Proteins were detected using the ECL Plus kit (Amersham). To test the mAb 9E10, stable cell lines expressing the c-myc tagged GBV-C E2 glycoprotein were collected and lysed using Laemmli buffer.

At a 1 in 2000 dilution the anti-His antibody showed low nonspecific staining, but good staining of the 122kDa His tagged control protein. Similarly, the monoclonal antibody 9E10 reacted specifically with low background at a 1 in 700 dilution with the GBV-C E2 glycoprotein, constitutively expressed in HEK293. Figure 3.8 illustrates the 122kDA (lane 1) and the 40kDa (lane 2) protein bands, representing His-β-gal and the GBV-C glycoproteins respectively.

However, several attempts to demonstrate recombinant expression of KSHV glycoprotein H and glycoprotein L in immunoblots failed. Although reactivity of the primary antibodies was repeatedly demonstrated for the positive controls, the desired protein band could not be observed.

3.3 Sequencing of promoter region and insert

Recombinant expression of both viral glycoproteins could not be detected with any assay performed, indicating either extremely low levels of protein or mutations within the DNA sequences. Furthermore, colleagues reported similar problems in expression studies when using vector pcDNA3.3p6. This may indicate a general problem of the expression vector itself, rather than mutations leading to frame shift or stop codons within the coding region.

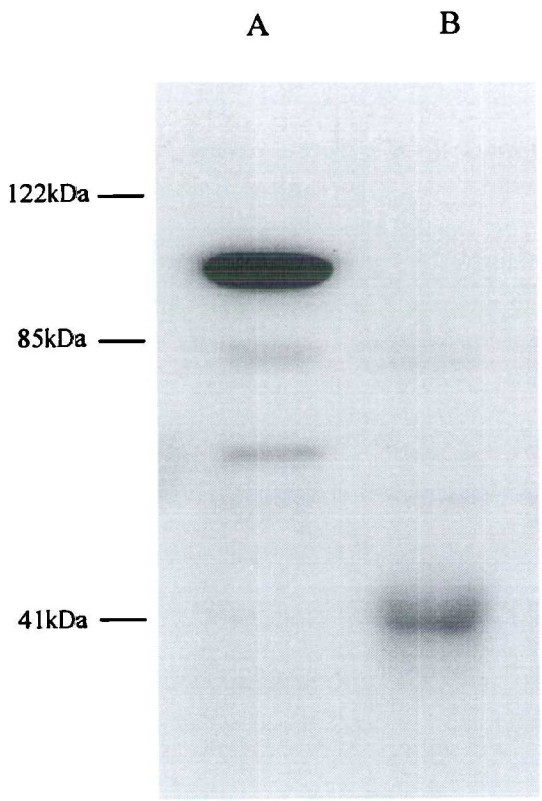


Figure 3.8: Testing of antibody activity. 293HEK cells were harvested and cell lysates prepared. Samples were separated on 12% SDS-polyacrylamide gel and analysed by Western blot using the penta-histidine antibody (Qiagen) and the 9E10 antibody (anti-c-myc). (A) 293 cells transiently transfected with pcDNA3.1-his-β-gal; probed with the penta-his monoclonal antibody. (B) 293 stable cell line expressing the c-myc tagged GBV-C E2 glycoprotein; probed with the c-myc monoclonal antibody 9E10.

Several pcDNA3.3p6 constructs were sequenced using a primer for the promoter region (pCMV) and an anti sense primer (p3rev$^-$) specific of vector sequences downstream of the insert. Primer sequences are listed in chapter 2.2. All sequences obtained were aligned and analysed for possible mutations.

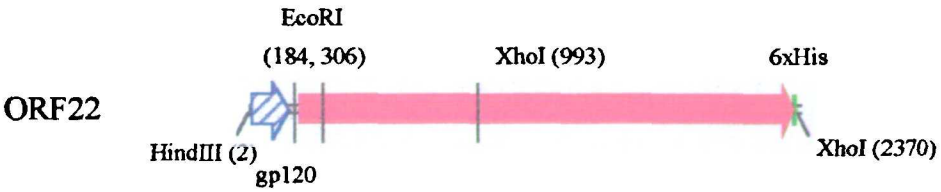
3.3.1 Promoter region

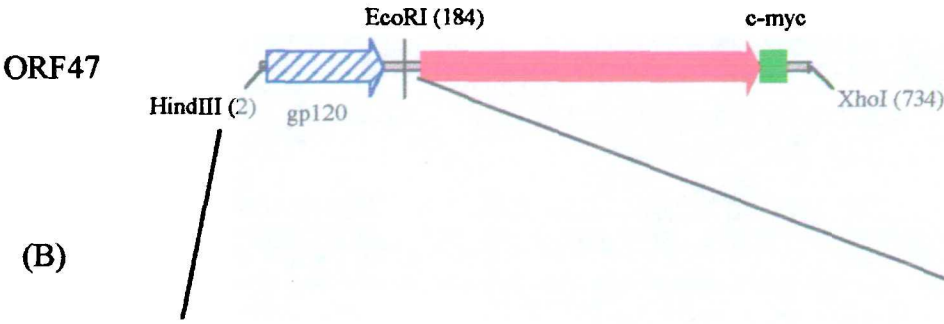
The promoter regions of each clone sequenced were identical with the sequence obtained from GenBank for the hCMV MIE enhancer-promoter as well as for the 5'untranslated intron (Accession number M21295) (data not shown). Therefore the low level or lack of expression was not due to mutations within the promoter region.

3.3.1.1 tPA leader sequence and glycoprotein coding region

DNA fragments encoding KSHV gH and gL were subcloned into the expression vector pcDNA3. Plasmids were digested with HindIII and NotI restriction enzymes after confirming that no existing restriction sites occurred within the coding sequences. Following bi-directional sequencing using pCMV and p3rev$^-$ sequences were aligned and compared to GenBank sequences. Unexpectedly, several minor and major mis-matches could be detected which were responsible for the lack of expression. Figure 3.9A illustrates schematically the HindIII/XhoI fragments found in pcDNA3.3p6 constructs. In addition the corresponding DNA and amino acid sequences identified are shown, including position 1 (HindIII site) to 250 (figure 3.9B). Results are identical for both KSHV open reading frames. To

(A)





(B)

```
+1        P  I   P  T  A   H  F  F   S  L  H  H   S  S  L
   HindIII
   ~~~~~~
1  AAGCTTCCTA TTCCCACTGC TCATTTTTTC TCTCTGCACC ACTCTTCTCT
   TTCGAAGGAT AAGGGTGACG AGTAAAAAAG AGAGACGTGG TGAGAAGAGA

+1   C  L  G   G  C  Y  S   *  W  F   N  F  Y   S  F  I
51 TTGCCTTGGT GGGTGCTACT CCTAATGGTT CAATTTTTAC TCCTTTATAT
   AACGGAACCA CCCACGATGA GGATTACCAA GTTAAAAATG AGGAAATATA

+1 F  I  *  F   T  S  P   I  V  P  H   I  S  S   S  R  S
101 TTATATAATT CACTTCTCCA ATTGTCCCTC ATATCTCCTC CTCCAGGTCT
    AATATATTAA GTGAAGAGGT TAACAGGGAG TATAGAGGAG GAGGTCCAGA

+1  E  D  P  L   V  T  A   A  S  V   L  N  S  N   *  R  S
                                     (G) EcoRI
                                     (C) ~~~~~~
151 GAAGATCCAC TAGTAACGGC CGCCAGTGTG CTGAATTCCA ATTGAAGGTC
    CTTCTAGGTG ATCATTGCCG GCGGTCACAC GACTTAAGGT TAACTTCCAG

+1   F
201 GTTT } ORF22 (nt 37182 to 39300)
    CAAA } ORF47 (nt 69433 to 69855)
```

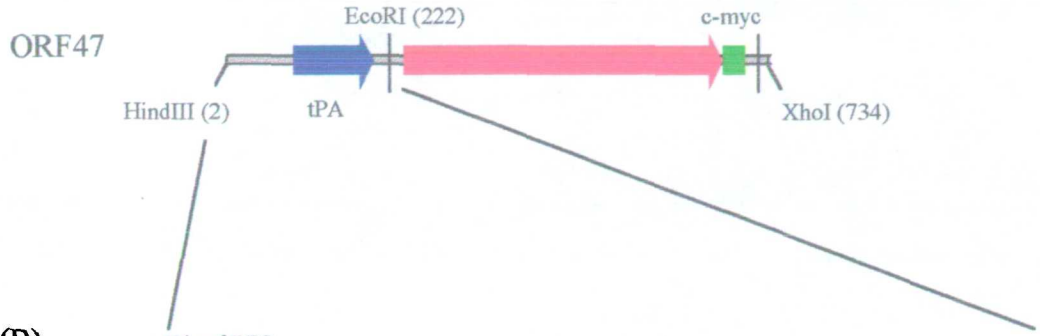
Figure 3.9: Schematic picture of the HindIII/XhoI fragment identified in the pcDNA3.3p6 constructs (A) and the corresponding nucleotide sequences between the HindIII site and the first nucleotide sencoding the respective KSHV open reading frame (B). Blue patterned arrows/letters indicate the 3' end of HIV-1 gp120; red arrows/letters symbolise the KSHV coding region; green blocks show the protein tags;letters in brackets highlight the deletion. Stars represent stop codons.

highlight the difference between the sequences found and the sequences expected, a schematic picture and the respective DNA sequences encoding a tPA leader sequence linked to the KSHV open reading frames are shown in figure 3.10.

3.3.1.1a Leader sequence of the tissue plasminogen activator

The tPA leader sequence was supposed to be in frame with the coding sequence of the respective KSHV reading frame. However, a major mis-match has been identified upstream of the glycoprotein coding region; between the HindIII site and the first nucleotide of the glycoprotein coding region (figure 3.9b, black letters). Sequence alignments with the original tPA leader sequence revealed that no tPA leader sequence was present. The polylinker of the vector as well as the KSHV open reading frame could be identified but the region between the HindIII restriction site and the first nucleotide corresponding to the viral sequence were unknown. Furthermore, within this region no start codon in frame with the insert could be detected. BLAST searches showed that the 155bp unknown region is identical with the 3'end of HIV-1 $gp120_{HXB2}$, nucleotide 7776 to 7621 respectively (GenBank accession number M38432). This indicates that the constructs contain sequences of KSHV glycoproteins linked to the C terminus of gp120, instead of the tPA leader sequence. Although EcoRI/XhoI digests had been performed this mistake has not been detected initially. Schematic pictures in figure 3.9A and 3.10A demonstrates the HindIII/XhoI fragment with the gp-120 region and with the tPA leader sequence. Regarding EcoRI and XhoI restriction sites, there is no major difference between the two sequences, indicating that a simple digest would

(A)



(B)

```
        HindIII
        ~~~~~~
  1  AAGCTTCTGA GCACAGGGCT GGAGAGAAAA CCTCTGCGAG GAAAGGGAAG
     TTCGAAGACT CGTGTCCCGA CCTCTCTTTT GGAGACGCTC CTTTCCCTTC

 +2                                                M   D   A   M
 51  GAGCAAGCCG TGAATTTAAG GGACGCTGTG AAGCAATCAT GGATGCAATG
     CTCGTTCGGC ACTTAAATTC CCTGCGACAC TTCGTTAGTA CCTACGTTAC

 +2  K   R   G   L     C   C   V     L   L   L     C   G   A   V     F   V   S
101  AAGAGAGGGC TCTGCTGTGT GCTGCTGCTG TGTGGAGCAG TCTTCGTTTC
     TTCTCTCCCG AGACGACACA CGACGACGAC ACACCTCGTC AGAAGCAAAG

 +2    P   S   Q     E   I   H   A     R   F   R     R   G   A     R   S   T
151  GCCCAGCCAG GAAATCCATG CCCGATTCAG AAGAGGAGCC AGATCCACTA
     CGGGTCGGTC CTTTAGGTAC GGGCTAAGTC TTCTCCTCGG TCTAGGTGAT

 +2 S   N   G   R     Q   C   A     G   I   P   I     E   G   R     L   T   C
                              EcoRI
                              ~~~~~~
201  GTAACGGCCG CCAGTGTGCT GGAATTCCAA TTGAAGGTCG TTTGACATGT
     CATTGCCGGC GGTCACACGA CCTTAAGGTT AACTTCCAGC AAACTGTACA
```

Figure 3.10: Schematic picture of the HindIII/XhoI fragments, assuming a tPA leasder sequence linked to the KSHV open reading frame (A). Corresponding nucleotide sequences spanning the HinIII site and the first nucleotide encoding the respective KSHV open reading frame are shown in (B). Blue arrows/letters indicate the tPA leader sequence; red arrows/letters symbolise the KSHV coding region; green blocks show the protein tags.

hardly have shown a different fragment pattern. The 'gp-120' sequence exhibits no additional EcoRI or XhoI restriction site. A plasmid containing gp120-gH would also result in a linearised form of the vector, a 120bp, a 690bp and a 1380bp DNA fragment. A digest using gp120-gL plasmid constructs would also only release the glycoprotein coding region. Looking at the corresponding DNA sequences and amino acid sequences spanning this particular region (figure 3.9B) an interesting observation can be made. A putative initiation ATG motif cannot be detected upstream of the coding region of the KSHV glycoproteins. Thus it is impossible to produce recombinant KSHV glycoprotein H or glycoprotein L by using these particular plasmid constructs. These findings indicate that restriction digest and ligation procedures leading to the genesis of vector pcDNA3.3p6 have not been performed correctly, as were the further experiments to evaluate the activity of this plasmid. In summary, the recombinant expression of gH and gL using this particular vector is impossible. The engineered C terminal protein tags will be transcribed either out of frame or early termination of transcription will occur. Besides the non-existent tPA leader sequence a further single base deletion of guanine in position 221 or 222 (see figure 3.10B) was identified; indicated by the letters in brackets in figure 3.9B (numbering with reference to the HindIII restriction site in position 1). Assuming the tPA leader sequence, this mutation would have caused a frame shift or early termination of protein translation due to a stop codon. This deletion alone would have made it impossible to detect the engineered C terminal protein tags. Interestingly, the deletion is next to the EcoRI

site that was involved in the clone Amp cloning strategy using uracilated extension at the vector.

3.4. Summary and Discussion

The herpesviral glycoprotein H can be found in any known herpesvirus, usually in association with a second viral glycoprotein, gL. Studies have revealed that gH plays an important role in viral penetration into the host cell as well as functions in cell to cell fusion (Browne *et al.* 1996; Wilson *et al.* 1994; Anderson and Gompels, 1999). Recently a new gamma herpesvirus, KSHV, has been identified and implicated to be important in KS pathogenesis. Protein homology searches of KSHV encoded proteins have identified ORF22 and ORF47 as gH and gL counterparts. Open reading frame 22 exhibits features of a transmembrane protein including a hydrophobic sequence at the N terminus, that may serve as a signal sequence and a hydrophobic stretch near the C terminus that may indicate a membrane spanning domain. Furthermore the glycosylation site NGTV, close to the hydrophobic domain is also present in KSHV ORF22/gH at residues 688 to 691. This motif is highly conserved within the family of herpesviridae (McGeoch *et al.* 1995). Although this site for N-linked glycosylation is well conserved, its function is unclear. Nevertheless, the NGTV motif within ORF22/gH supports its classification as a gH homolog. KSHV ORF47 also encodes a potential N terminal signal sequence, but lacks a hydrophobic region that could function as a transmembrane domain. To characterize these two viral glycoproteins in detail we were interested in the establishment of stable mammalian cell lines. Since the authentic production of glycoproteins is dependent on post-translational modification the use of a mammalian expression system appeared to be advantageous. As described before the expression of HIV glycoprotein gp120 have shown that the replacement of the natural N-terminal signal sequence by the

tissue plasminogen activator (tPA) signal peptide can result in an increase of glycoprotein production. Consequently DNA sequences encoding KSHV ORF22/gH and ORF47/gL were cloned in frame with a tPA leader sequence of the vector pcDNA3.3p6. Transient transfection experiments and the establishment of a cell line constitutively expressing the glycoproteins proved to be unexpectedly difficult. Despite relatively high transfection levels, as judged by a β-gal control plasmid and the successful selection of PCR positive cell lines, several immunological methods failed to detect recombinant protein. Indirect EIA procedures gave evidence of a low amount of protein, although those results were inconsistent. Explanations of a low expression could have been due to several problems: firstly, toxicity of the recombinant protein causing cell death. Cells are forced to switch off glycoprotein synthesis for their own survival. Secondly the vector design may not be suitable for the expression of these particular glycoproteins; thirdly, the engineered C-terminal modifications are expressed out of frame or degraded soon after production and therefore detection using monoclonal antibodies specific for those tags is impossible. Finally, gH and gL might elucidate a low immune response resulting in a low titer of specific antibodies when human serum is used. The vector pcDNA3.3p6 has been used in other expression studies and was reported to produce reasonable amounts of protein that can be detected by EIA and western blot. It was also unlikely that the lack of expression was due to mutations or frame shift events as different products were amplified from different sources. We therefore believed that the two glycoproteins may only be produced in low amounts and sensitive methods are

necessary to demonstrate expression. However, since EIA procedures were also unsuccessful, we were suspicious that during vector generation, the promoter sequence may have been altered. We decided to sequence part of the vector, including the promoter region together with the insert. Although colleagues repeatedly assured that this vector produces recombinant protein we made an unexpected discovery. The tPA leader sequence could not be detected, instead sequences encoding the 3' end of HIV-1 gp120 were identified, missing a start codon in frame with the KSHV fragments. This major error is most likely to have occurred during generation of the vector. Briefly, the vector pcDNA3.3p6 is based on other mammalian expression vectors, namely P2 and pEE6.neo. For a description of these vectors see chapter 2.1. and the plasmid maps in Appendix II. The promoter in pcDNA3.3p6 is derived from pEE6.neo as a BglII/HindIII fragment inserted into the polylinker of the pcDNA3 vector. The tPA leader sequence was obtained from the P2 plasmid that encodes tPA-HIV-1 gp120. To obtain the tPA leader sequence the vector P2 had to be digested with HindIII and BglII, producing several fragments of similar size, including the tPA leader sequence and a 3'gp120 fragment. The pcDNA3 vector containing the pEE6 promoter was then linearised using HindIII and BamHI. A simple ligation reaction containing the linear form of the vector and the BglII/HindIII insert were subsequently performed. However, these ligation reactions must have been performed using the gp120 fragment as insert instead of the tPA leader sequence, thereby producing a vector with similar size to the expected one with the same polylinker. Simple agarose gel analysis would not have identified this mistake. A

proper restriction analysis using an enzyme, e.g. Bsp1286I that allows discrimination between the gp120 fragment and the tPA leader sequence (a Bsp1286I site is only present in the tPA leader sequence) would have identified the mistake. Furthermore, sequencing the insert would have been useful. However, apart from this major mis-match another mutation has been identified. This single base deletion immediately preceding the EcoRI site would also cause early termination of protein translation, whether or not the tPA leader sequence was present. Regarding the location of this deletion the cloning procedure, in particular the use of the uracilated tails, may not have been optimized and resulted in point mutations due to the enzymes activity. In summary, the vector pcDNA3.3p6 is defective and cannot be used for any expression studies. However, the detection procedures developed in this chapter can be applied to further expression studies in order to assess protein expression using a different vector. To this end, the appropriate DNA sequences were re-amplified in full-length from different clinical material and inserted into a commercially available mammalian expression vector.

4. Recombinant expression of KSHV glycoprotein H and glycoprotein L in two different cell lines

4.1 Introduction

Expression of the KSHV ORF22/gH and ORF47/gL was unsuccessful using the expression vector pcDNA3.3p6. The series of experiments described in chapter 3 demonstrated that the lack of expression was due to the vector itself rather than the viral sequences amplified. However, it is still of interest to determine if KSHV gL interacts as a chaperone for gH folding and mediates cell surface expression of gH, as described for other herpesviruses (see proceeding chapter). To test this hypothesis we were interested in the intracellular distribution of gL under two circumstances: (a) when individually expressed and (b) in combination with plasmids encoding the KSHV gH homolog. Glycoprotein L has been demonstrated to be similarly dependent on gH for its cell surface localization. When expressed alone, the protein is either secreted into the culture medium (HSV-1, HCMV) or retained within the cytoplasm (EBV, VZV) (Westra *et al.* 1997; Kaye *et al.* 1992; Pulford *et al.* 1995; Duus and Grose, 1996). To study the characteristics of the KSHV gH and gL counterparts, viral open reading frames from several patient samples were amplified and cloned into the appropriate restriction sites within the polylinker of the commercially available vector pcDNA3.1 (Invitrogen). Two different cell lines, the epithelial cell line HEK293 and the fibroblast cell line Cos7, were transfected with these plasmid constructs and analysed for protein expression. Briefly, protein expression was analysed with different procedures, varying in detection sensitivity. Overall, expression levels

were low in both cell lines and this complicated further analysis of the cellular localization of each glycoprotein.

However, we have obtained clear evidence that the surface location of ORF47/gL is dependent on the presence of the ORF22/gH gene product.

4.2 The vector pcDNA3.1 and the choice of mammalian cell lines

The vector pcDNA3.1zeo is commonly used for the expression of recombinant proteins in mammalian cells. A detailed vector description is given in chapter 2.1 and a schematic plasmid map is included in Appendix II. An hCMV immediately early promoter drives high-levels of expression and the zeocin resistance gene enables selection for stable cell lines in mammalian cells.

As described in chapter 3, the authentic expression of glycoproteins is dependent on the competence of the cell line to perform post translational modification. Based on extensive experiments with HIV gp120, one cell line of choice is the human embryonic kidney cell line HEK293. Also to be considered is the possible low amount of protein synthesized. This problem may be overcome by using the African green monkey cell line Cos7 in combination with pcDNA3.1 constructs (see chapter 2.1). This cell line encodes the SV40 large T antigen, allowing vectors encoding the SV40 origin of replication to replicate as an episome to a high copy number within cells (Chittenden *et al.* 1991). This may increase the amount of protein produced per cell and be an advantage for ORFs with low expression levels (Harvey *et al.* 1997).

4.3 Selection of patient samples

Several patients who had been diagnosed with a KSHV associated disease, including classical KS, HIV-associated KS and primary effusion lymphoma, were selected. A non-patient material, tissue culture supernatant from KSHV positive BCP-1 cells, was included in the study as a control and will be designated as sample number three. Five patients were selected and total DNA extracted from blood samples using the Qiagen Blood Kit. Two samples failed to amplify, possibly due to DNA degradation while stored. The remaining three samples were included in the study and both gH and gL open reading frames cloned into the mammalian expression vector. Briefly, patient 4 is a 14-year old girl, infected with HIV who showed subcutaneous nodular KS lesions. This patient was the subject of a study investigating the anti-KS activity of the drug thalidomide (Soler *et al.* 1996). However, the DNA sample used for our expression studies was obtained before this treatment started.

Patient number 5 is a 40-year old male with late stage AIDS and primary effusion lymphoma. The cell line SY, used for KSHV infection studies described in chapter 6, is derived from ascetic fluid of this patient. Finally, patient number 7 is an elderly patient with history of classic KS.

4.4 Cloning of full-length KSHV ORF22/gH and ORF47/gL

4.4.1 Amplification of the respective sequences by nested PCR

Oligonucleotide primers for nested PCR were designed to amplify the complete sequences of KSHV ORF22/gH and ORF47/gL (see section 2.2.2.1). To achieve optimal protein expression levels several nucleotide motifs were included in each

primer. For cloning purposes the restriction sites HindIII and NotI were introduced into the sense and anti sense inner primer respectively, after verifying that no possible sites for these enzymes occurred within the coding sequences. Throughout higher eukaryotes the 5' non-coding sequence, CCACCATGG has been described to play an important role in the recognition of ribosomes (Kozak, 1987; Kozak, 1984). Most crucial is the purine, at position -3 (mostly an A, though G may occur), the G residue immediately following the AUG codon also influences expression. The 5' non-translated Kozak sequence motif, CCACC, was introduced immediately upstream of the AUG codon of the respective open reading frames. Given these changes in the primer sequences the nested amplification of full length KSHV ORF22/gH and ORF47/gL required a new set of optimizations of PCR conditions (data not shown). An optimal amplification result could be achieved by applying the PCR conditions detailed before for truncated forms of the respective viral ORF (see section 3.1.2.3).

4.4.1.1 Full length ORF22/gH

The amplification of full length ORF22/gH produced a 2,230kb fragment. To avoid possible polymerase induced errors, the enzyme Expand™ (Boehringer Mannheim) was used, exhibiting proof reading abilities. One microliter of the sample were added to a 50µl PCR reaction mixture and reactions carried out under a variety of amplification conditions, mainly differing in oligonucleotide concentration (200 and 400µM) and annealing temperature, ranging from 44°C to 56°C. Four samples (patient 3, 4, 5, 7) out of six could be detected using the

conditions detailed in section 2.2.2.2 (54°C, 200μM), but the remaining two failed to produce the desired fragments under any of the conditions tested. Amplified ORFs of each patient are demonstrated in figure 4.1, lanes 1 to 4.

4.4.1.2 Full-length ORF47/gL

The ORF47/gL is smaller in size (600bp) than ORF22/gH and therefore the conditions described for the amplification of ORF47/gL in section 3.1.2.3 were successfully applied. Each patient sample was amplified and synthesized amplicons are shown in figure 4.1 lanes 5 to 7. As observed for ORF22/gH patient one and two again failed to amplify, suggesting possible degradation of the template DNA within these samples.

4.4.2 Cloning and verification of the glycoprotein coding sequences

The amplified DNA fragments were agarose gel purified and digested with the enzymes HindIII and NotI. The vector pcDNA3.1 was linearised using the same enzymes and dephosphorylated using shrimp alkaline phosphatase (SAP). Following ligation and transformation bacterial colonies were analysed by PCR and positive transformants selected for over-night culture. From each patient five clones were chosen and plasmids purified. To verify the respective inserts each plasmid was digested with HindIII and NotI, and then sequenced with the oligonucleotide primers pCMV and p3rev. As expected, restriction enzymes released the insert leaving the vector in linearised form (data not shown). Three plasmids showing the right fragment pattern were selected and further sequenced

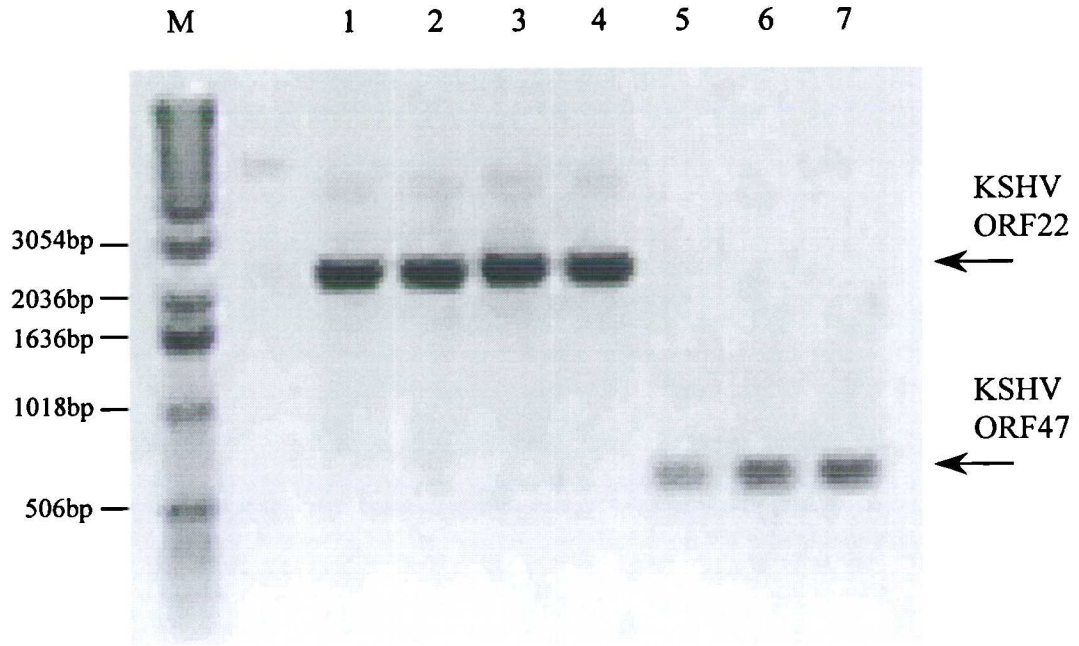


Figure 4.1: PCR amplification of KSHV ORF22 and ORF47. Total DNA has been extracted from different patient samples and one microliter was applied to PCR. Amplicons were separated on a 1% agarose gel.
M: 1kb DNA ladder (Life Technologies)
Lane 1: ORF22 from BCP-1 supernatant, designated as sample number 3
Lane 2: ORF22 from patient 4
Lane 3: ORF22 from patient 5
Lane 4: ORF22 from patient 7
Lane 5: ORF47 from BCP-1 supernatant, designated as sample number 3
Lane 7: ORF47 from patient 4
Lane 8: ORF47 from patient 5

to confirm the presence of the Kozak sequence and the start codon in frame with the glycoprotein coding sequence as well as the C terminal protein tag. All ORF47/gL constructs were sequenced in full, whereas for ORF22/gH only 350bp at the 5' and 3' end were analysed.

In summary, coding sequences were all in frame with the Kozak sequence and the ATG start codon. Sequences encoding ORF47/gL were in frame with the c-myc epitope and no differences from the published KSHV sequences were observed. Plasmid constructs containing ORF22/gH were in frame with the Kozak sequences and the initiation motif ATG. At the 3' end the nucleotide motif for the histidine residues (ORF22/gH) could be detected, together with the stop codon. The schematic picture in figure 4.2 illustrates the inserted glycoprotein coding sequences within pcDNA3.1.

Plasmids were named with respect to the inserted sequences (gH or gL), the patient number and the number of the clone selected. For example, ORF22/gH derived from patient number 4 was labeled pcDNA3.1gH4.6; number six, being the clone selected.

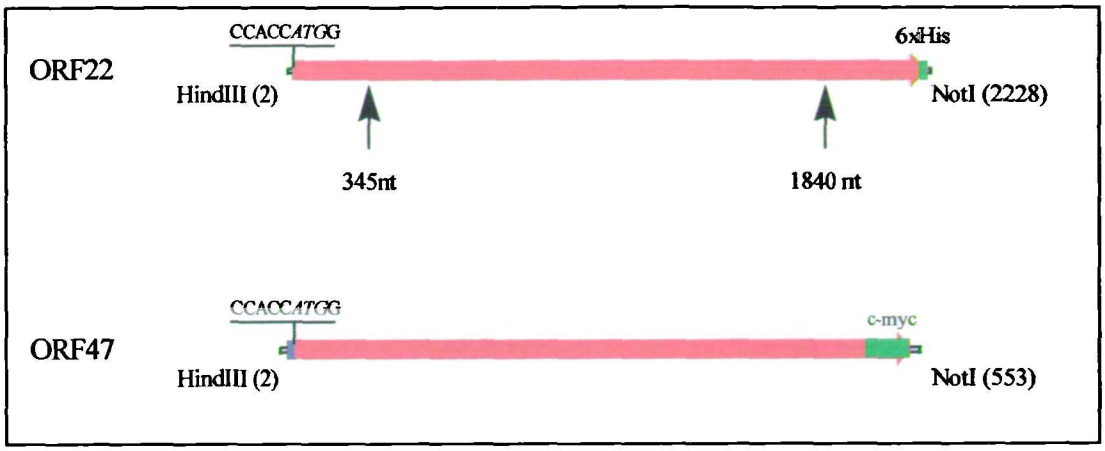


Figure 4.2: Schematic diagram of KSHV ORF22/gH and ORF47/gL inserted into the HindIII/NotI polylinker of pcDNA3.1. Sequence motif (blue color) indicates the Kozak sequence; red arrow: glycoprotein coding sequence; green: protein tag. The arrows in the upper figure indicate the ends of the regions of ORF22/gH sequenced, (ORF47/gL was sequenced in full). Numbers below the arrows refer to nucleotide position.

4.5 Recombinant expression of KSHV ORF22/gH and ORF47/gL

Recombinant expression studies using full-length constructs of KSHV ORF22/gH and ORF47/gL, can be subdivided into three parts. Firstly, transient transfection experiments tested the level of expression of each plasmid; secondly, larger scale transient transfections using larger amounts of cells were analysed by western blot to estimate the molecular mass of the protein. Finally, stable cell lines were established that produced constitutively the protein of interest, allowing a more precise in vitro analysis. In this case transient as well as stable expression levels were low, requiring sensitive detection methods, including indirect immunofluorescence assay (IFA) and flow cytometry. Furthermore, the influence of sodium butyrate on the expression level of transfected cell lines was examined.

Throughout these experiments ORF22/gH could only be demonstrated using certain detection procedures, including western blot and flow cytometry. This was ascribed to inconsistent results produced by the monoclonal antibody "penta-his", even when using the control protein his-β-galactosidase (see chapter 3.1, section 3 and 4.2). The data obtained will be discussed in more detail below.

4.5.1 The 293HEK cell line

As described in chapter 3, transient transfections allow a high level of expression within a short time period, making this procedure ideal for initial expression experiments. ORF47/gL plasmids were tested for their level of expression in "blue cell assays" (described in chapter 3), once expression was demonstrated the plasmid was used for further experiments. Detection of the ORF22/gH gene product was only visible by western blot procedures. Using the control plasmid pSEC-GBV-C E2 (see Appendix II for plasmid map) in duplicate transfections, staining with the monoclonal antibody 9E10 for the c-myc epitope indicated high levels of transfection and expression, whereas using the penta-his monoclonal antibody in IFA no specific fluorescence could be observed (data not shown). A similar result was obtained when detecting expression of the control protein his-β-galactosidase by flow cytometry (see below). As a consequence the detection of ORF22/gH was limited to western blot methods and hence the distribution of recombinant KSHV gH within the cell could not be directly established using this particular antibody. However, as discussed later in this chapter, ORF22/gH can be identified indirectly when co expressed with ORF47/gL, assuming an interaction

between those two glycoproteins. This situation also applied to the expression studies using COS7 cells discussed later in this chapter.

4.5.1.1 Optimization of transient transfections using a reporter plasmid

A new transfection reagent was available, Effectene (Qiagen), that was claimed to result in very high transfection efficiencies. Effectene is a non-liposomal lipid reagent that transfers DNA into mammalian cells in combination with a special DNA-condensing reagent, named 'enhancer'. Optimal transfection is influenced by several parameters, including the DNA-effectene ratio, the DNA- enhancer ratio, the type of cell line used and the plasmid construct that is to be transfected. Initially, cells were transfected with the control plasmid pcDNA3.1-his-β-Gal as the detection of the β-galactosidase is simple and less time consuming, but would give a rapid answer about the response of HEK293 cells to the transfection procedure.

HEK293 cells were transfected in 60 mm dishes according to the 'pipetting scheme' given by the manufacturer. Different amounts of DNA (0.5μg, 1μg and 2μg) were transfected and the enhancer and effectene adjusted with respect to these DNA concentrations. Exact DNA-Effectene ratios are given in the legend of figure 4.3. Following 48h of incubation, cells were harvested for western blot as described in chapter 3 using Laemmli buffer followed by sonication. A small cell patch was left in the dish to stain for β-galactosidase expression using X-gal. Lysates were separated on a 7% SDS-PAGE, approximately $2x10^5$ cells being

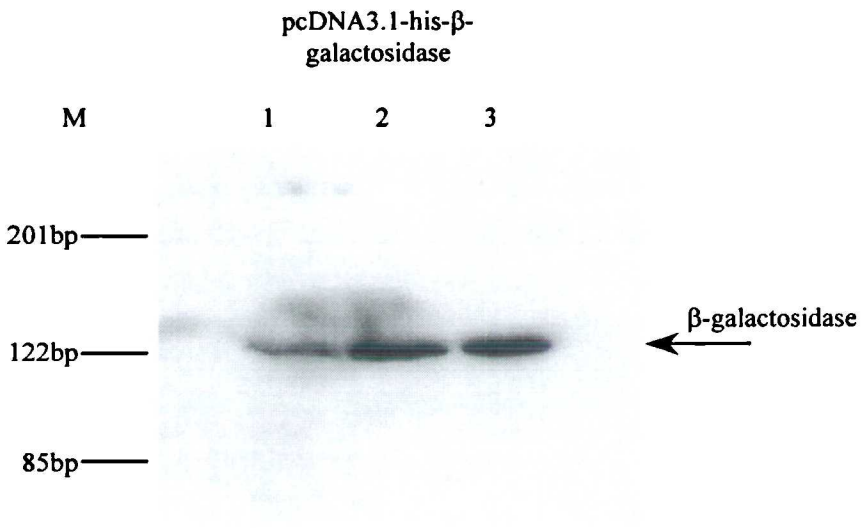


Figure 4.3: Optimization of transient transfection using effectene as a transfection reagent. The control plasmid pcDNA3.1-his-β-galactosidase was transfected into HEK293 cells under various conditions. After two days incubation cells were harvested, lysed in Laemmli buffer and analysed by (7%) SDS-PAGE. PVDF membranes were probed with the Mab penta-his and a goat-anti-mouse-secondary HRP-conjugated antibody.
M: Molecular weight marker, Rainbow marker (Amersham)
Lane 1: 0.5μg DNA; 4μl enhancer; 12.5μl effectene (1/25 DNA-effectene ratio)
Lane 2: 1μg DNA, 8μl enhancer; 25μl effectene (1/25 DNA-effectene ratio)
Lane 3: 2μg DNA, 16μl enhancer; 50μl effectene (1/25 DNA-effectene ratio)

loaded per well. Proteins were transferred to PVDF membrane and stained for the presence of the histidine tagged protein.

Following transfection, proportional association between the effectene concentration and cell death could be observed; the more effectene, the more the cells died or their growth was inhibited. An optimal transfection has to compromise between this cell death and yields of protein. To reduce cell death, growth medium was replaced by fresh complete medium 24 hours post-transfection. In figure 4.3 the immunoblot demonstrates the amount of protein produced under various transfection conditions. An optimal result was obtained using 1μg or 2μg of DNA and effectene at a 1 in 25 dilution (lane 2 and 3). Although the β-galactosidase could be detected when using lower amounts of DNA, transfection was less efficient as judged by the intensity of the protein band. In addition, the cell patches remaining in the dishes were stained (data not shown), reflecting the western blot observation. To conclude, a high amount of effectene is necessary to transfer DNA into HEK293 cells. All further transfection experiments were performed at the concentrations used above (for 60mm dishes, 1μg DNA; 1 in 25 effectene ratio). In cases of smaller culture vessels or lower numbers of cells, e.g. in 12, or 24 well dishes, the transfection procedure was adjusted, respectively.

4.5.1.2 Expression levels of different gL plasmid constructs

Each plasmid construct was evaluated in transient transfections followed by simple histochemical staining procedures (blue cell assay) as previously described (chapter 3). The antibody penta-his failed to detect ORF22/gH recombinant

protein using this assay, although the control protein was known to encode histidine residues and known to be expressed and subsequent western blot experiments showed the ORF22/gH protein (see below). Consequently, only plasmid constructs encoding KSHV ORF47/gL were tested. Controls include the galactosidase plasmid, to monitor transfection efficiency, and a c-myc tagged control plasmid (pSEC-GBV-C.E2) (data not shown), together with cells that had been mock-transfected. Transfection was performed in 24 well dishes (0.3μg DNA; 1/50 DNA-effectene ratio) and cells fixed 48 hours post transfection. After 24 hours of incubation cells started to detach. The growth medium was replaced and the incubation continued, but cell loss was still high compared to the negative control at the time of methanol/acetone fixation. Following staining procedures, the differences in expression between the transfection controls and the plasmid constructs of interest was surprising. In figure 4.4, several tested plasmids are illustrated, indicating that gL 4.2 (A) and gL 5.10 (B) could be detected more readily than the other constructs. The number of blue cells produced by the gL encoding plasmids was low in comparison to the positive control (F; >50%), although clearly positive when compared to the negative HEK293 cells (E). On average, >5% of cells stained blue, while the positive control showed more than 50% transfection efficiency. The blue cell assay pictures also illustrate the decrease in cell growth, in that discrete patches of cells were formed instead of the uniform monolayer seen for cells transfected with pcDNA3.1-β-gal (figure 4.4F). Despite the low levels of expression this result demonstrates promoter activity and the ability of these plasmids to produce recombinant protein. There may be a

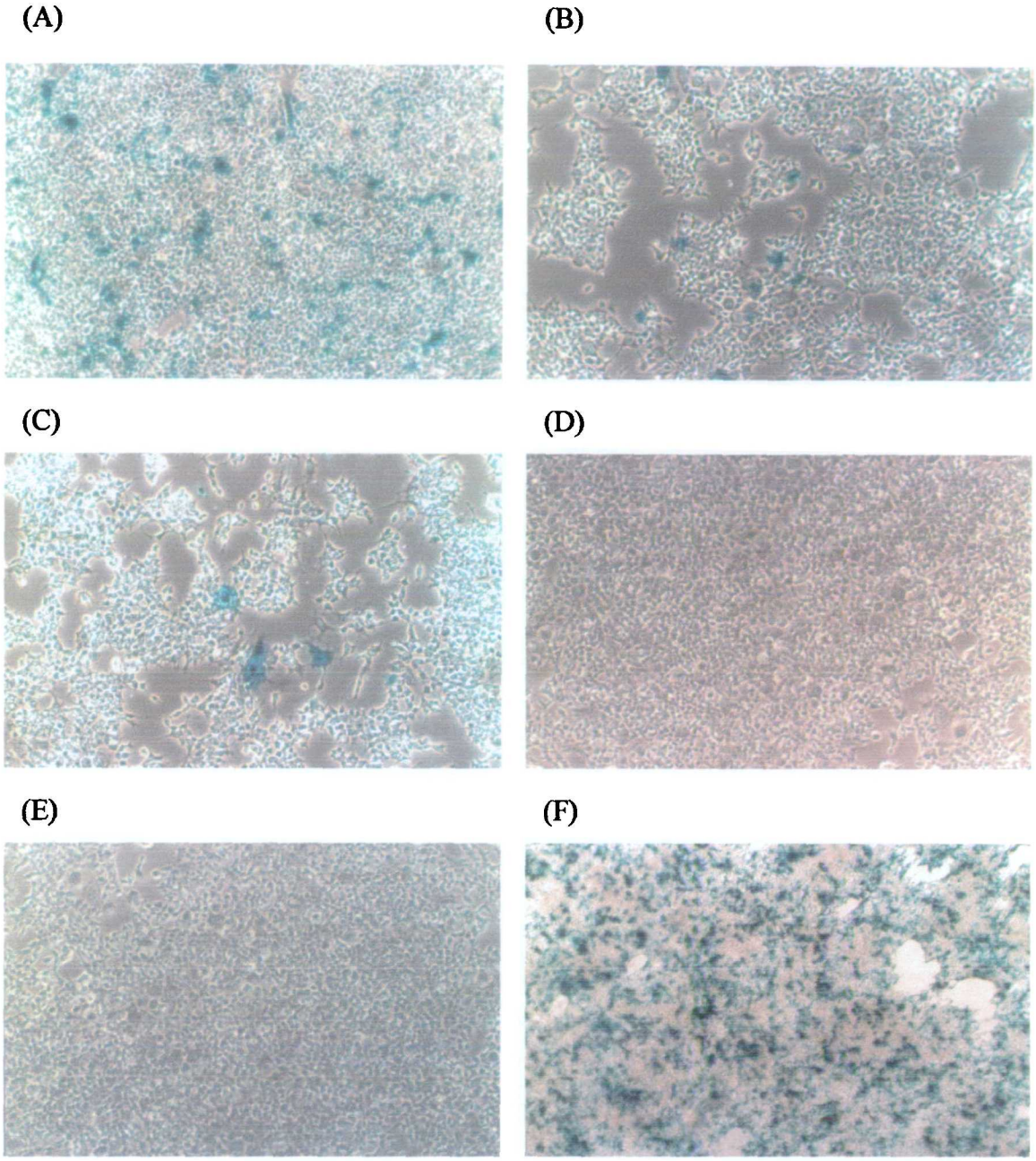


Figure 4.4: Analysis of the expression levels of different ORF47/gL plasmids in transient transfections. Plasmids were transiently transfected into 293 HEK cells and stained 48 hours post-transfection. Cells were fixed in methanol/acetone, incubated with primary mAb 9E10 and bound antibody was detected with a β-galactosidase conjugated anti-mouse secondary antibody.
A: pcDNA3.1-4.2gL
B: pcDNA3.1-5.10gL
C: pcDNA3.1-5.3gL
D: pcDNA3.1-3.2gL
E: Mock transfected cells
F: pcDNA3.1-β-galactosidase, positive control

possibility that the amount of protein could be increased by changing certain transfection parameters. Possibly the combination of transfection reagent and DNA may not be optimal, causing the cell death or inhibition of cell growth, seen after incubation. However, the lack of this problem for the two control plasmids used lead us to conclude that an ORF47/gL associated toxicity is a more probable explanation.

4.5.1.3 Re-evaluation of transfection procedure using the plasmid pcDNA3.1-4.2gL

To further improve the transfection conditions specifically for gL expressing plasmids, the plasmid pcDNA3.1- gL 4.2 was prepared in larger quantities. As described above, recombinant protein could be successfully detected when transfecting this plasmid into HEK293 cells. To establish transient transfections with high levels of recombinant protein but a low decline in cell number, various combinations of DNA and effectene were examined. Experiments were performed in both 60 mm and 24 well dishes, followed by western blot and blue cell assays. Although when effectene concentrations were lowered cell death was reduced, an increase in protein expression was not observed. Using 0.3μg DNA and a 1/50 DNA-effectene ratio staining procedures were most successful in 24 well dishes (data not shown). DNA concentrations greater than 0.3μg needed increasing amounts of effectene, but resulted in inhibition of cell growth and rapid detachment of cells from the surface. Furthermore, attempts to detect the glycoprotein in western blots using the monoclonal antibody specific for the c-

myc epitope (mAb 9E10) failed at any condition (data not shown). KSHV ORF47/gL is predicted to encode a 167 amino acid protein with an estimated size of 15kDa prior to post transitional modifications (>20kDa after glycosylations). Neither cell culture supernatant nor the soluble or the insoluble protein fraction showed any band migrating between 20 to 40kDa by western blot.

4.5.1.4 Reporter plasmid as an internal control shows high recombinant protein expression

All previous experiments indicated that the level of protein expression by plasmids encoding ORF47/gL is low in transient transfections. The reason behind this observation may be due to the toxicity of the protein itself or may be caused by the transfection method chosen. To monitor the transfection efficiency of a particular DNA sample, we decided to co-transfect the gL plasmid along with a reporter plasmid. The plasmid pcDNA3.1-his-β-galactosidase was selected, allowing for direct blue cell assay detection (via β-galactosidase) and western blot analyses (via histidine residues); for gL, expression plasmid 4.2 was chosen. HEK293 cells were transfected in 60 mm dishes and different ratios of the two plasmids assessed (control plasmid/gL: 1/1 and ¼). Cells were harvested 48 hours post transfection as described before. A small patch of cells was left in the dish, fixed in methanol/acetone and stained for β-galactosidase expression. Cell lysates were prepared by mixing harvested cells with an equal volume of Laemmli buffer followed by sonication and boiling. Cell supernatant or around 10^5 cells were run on 12% SDS-PAGE and analysed by western blot; firstly probed for ORF47/gL

expression with the monoclonal antibody 9E10 and secondly, after stripping the membrane, probed with penta-his. The corresponding protein band patterns are shown in figure 4.5.

4.5.1.4a Detection of the c-myc epitope (ORF47/gL-c-myc)

Unfortunately, a distinct cross-reactivity of the antibody with mock-transfected cell supernatant as well as cellular lysate (data not shown) could be observed (figure 4.5A). Western blot analyses showed in each lane, including the mock-transfected 293 sample, a protein migrating at approximately 66kDa. The estimated molecular weight of this protein may suggest fetal calf serum (FCS) as a target. In contrast, the c-myc positive control confirmed the activity of the antibodies and no cross-reactivity with FCS could be detected (data not shown). However, regarding the expression of ORF47/gL, no protein could be detected, between 20-40kDa. Unless the KSHV gL homolog may form a complex or associates with a cellular protein, these results confirm previous detection attempts. Namely, that transient transfections produce amounts of recombinant ORF47/gL lower than the detection limit of western blots.

4.5.1.4b Detection of the His-β-galactosidase expression

The histidine tagged β-galactosidase can be detected in two ways; histochemically using the substrate X-Gal or by immunoblot. As the membrane can be stripped western blot allows a direct comparison between the level of expression of the β-galactosidase and ORF47/gL for each sample.

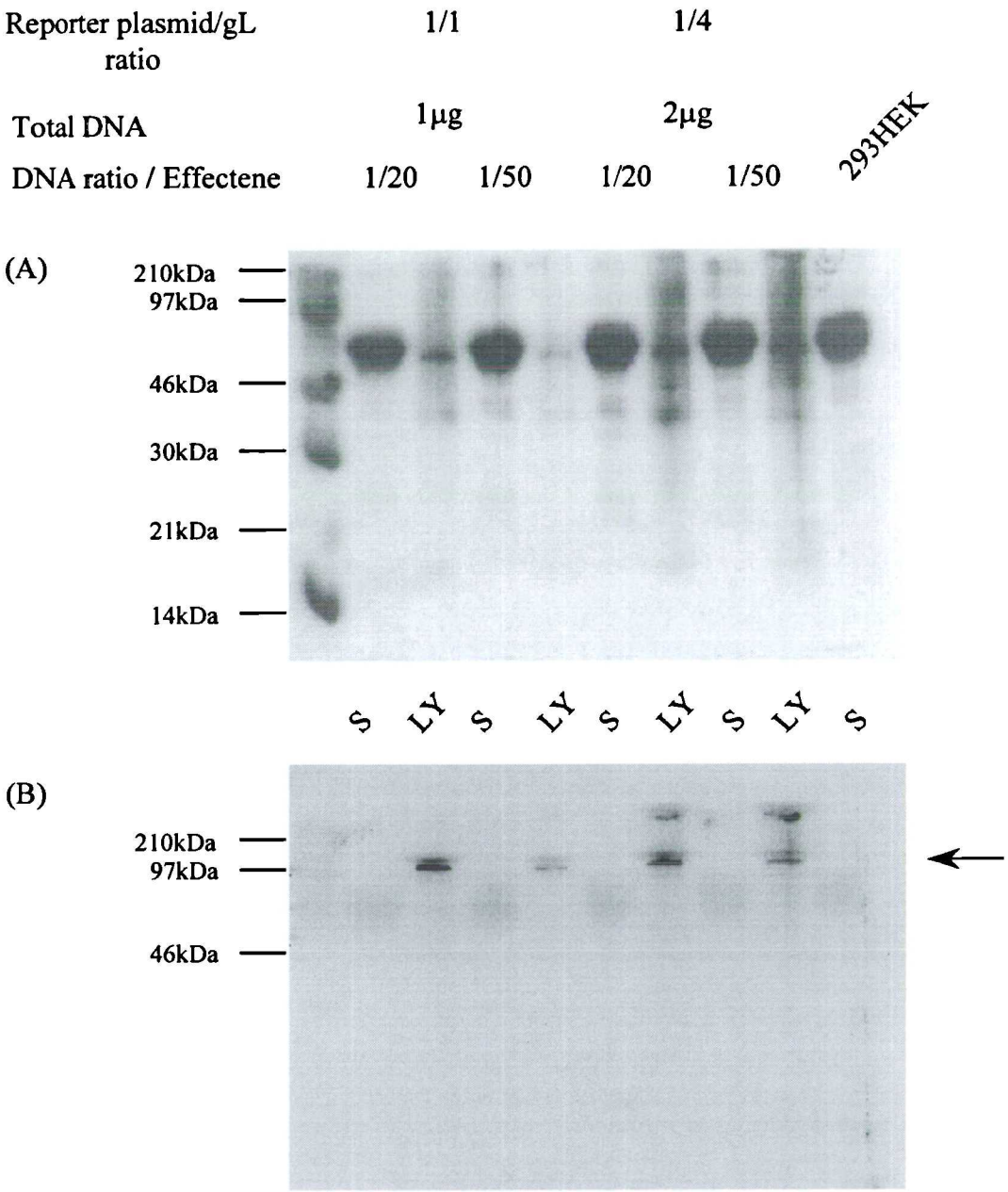


Figure 4.5: Western blot analyses of 293HEK cells transfected with both the reporter plasmid and pcDNA3.1-4.2gL under various transfection conditions (detailed above the picture). Lysates were obtained 48 hours post-transfection and subjected to 12% SDS-PAGE. ORF47 was detected by the Mab 9E10 (A) and the reporter protein his-galactosidase (see arrow) was identified with the Mab penta-his (B). As a negative control supernatant from mock-transfected cells was used. Numbers on the left are molecular masses in kilodaltons (kDa).
Supernatant (S) or lysate (LY) are indicated on the bottom of each lane

Blue cell assays showed a low number of positive cells, when compared to the control transfection (data not shown). This may be due to the presence of the second plasmid which prevents optimal transfer into the cell. Overall, the transfection efficiency is satisfactory and should enable protein detection in western blot procedures. Using the monoclonal penta-His the 120kDa β-galactosidase could be detected, (figure 4.5B) The protein is present in each lysate, although at a lower concentration than seen before (see figure 4.3). The intensity of the respective bands differ, but corresponded to the amount of blue cells detected. Evidently, the expression level of both plasmids is different, one protein being detectable in western blots (β-galactosidase) whereas the other seems to be missing. These results show that, using this transfection procedure, a sufficient amount of protein can be produced, as indicated by the reporter plasmid. This suggests that in transient transfection systems using 293HEK cells the recombinant expression of KSHV ORF47/gL only possible at levels undetectable by western blot procedures.

4.5.1.5 Transient expression of KSHV ORF22, the gH homolog

The second protein to be expressed is encoded by KSHV ORF22/gH and has an estimated molecular weight of 79kDa, prior to post translational modifications. Previous blue cell assays and indirect immunofluorescence assays showed that the detection of any histidine tagged protein using anti-histidine antibodies is unsuccessful, even for positive control proteins. Therefore the study of the ORF22/gH gene product was limited to western blot procedures.

Several different ORF22/gH plasmids (pcDNA3.1-4.6gH; pcDNA3.1-5.1gH; pcDNA3.1-5.2gH; pcDNA3.1-7.11gH) were transiently transfected into HEK293 cells using effectene and the conditions described for the control plasmid pcDNA3.1-his-β-galactosidase. During the transfection of ORF22/gH encoding plasmids an increase in cell detachment could also be observed. Cells were harvested 48 hours post-transfection and soluble/insoluble protein fractions separated along with cell culture supernatant as described in section 2.2.7.1 and analysed on a 12% SDS-PAGE followed by western blot (figure 4.6). A number of proteins which reacted with the penta-his antibody were found in the insoluble protein fraction of all the samples tested. The protein profile for each plasmid contained a group of reactive proteins ranging from 38 to 150kDa with a prominent band at 66kDa. The predicted molecular weight of ORF22/gH is suggested to be between 75 and 79kDa, without glycosylation. It is therefore possible that some bands seen in the western blot may correspond to incompletely glycosylated forms, breakdown products or the fully processed form of the ORF22/gH gene product. Their origin has not been determined as no antibody specific for KSHV ORF22/gH is available. However, lysate from mock-transfected cells showed a relatively similar pattern, as illustrated in figure 4.6; no. 5. There is no significant difference between lysate derived from mock-transfected or plasmid transfected cells. Given this observation, it is likely that the situation seen for ORF47/gL may apply to ORF22/gH as well. The amount of protein produced being too low to be detected by immunoblot.

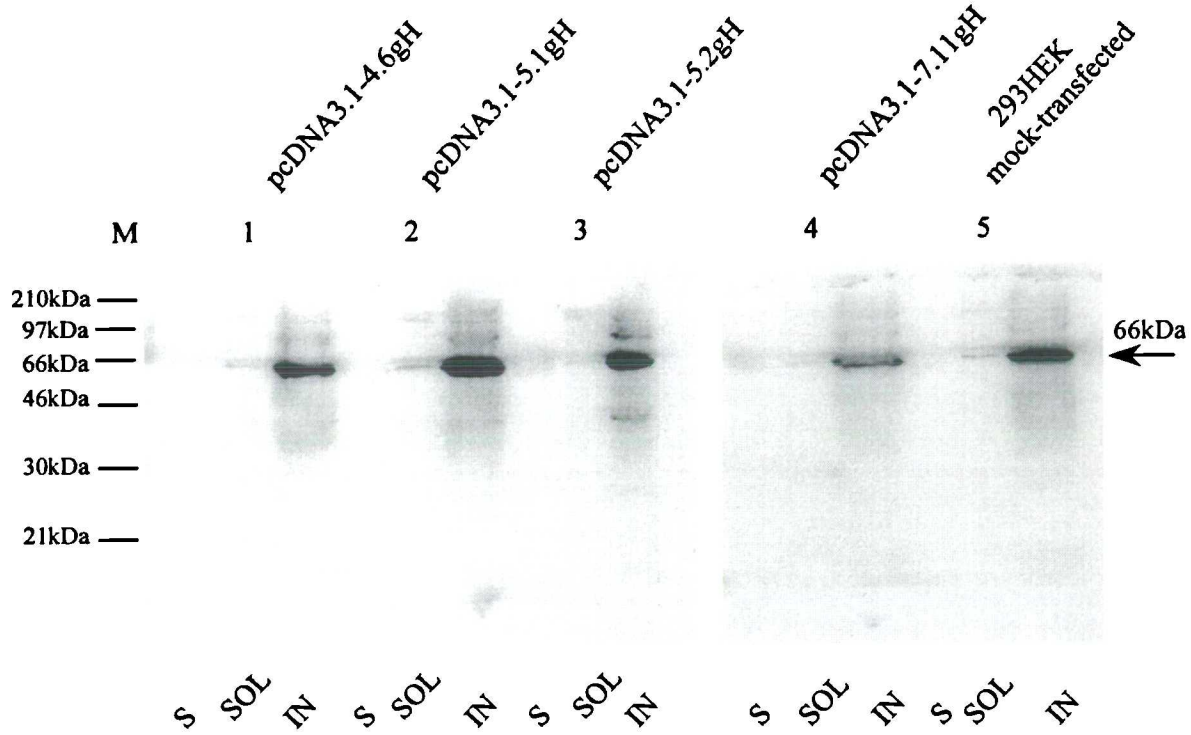


Figure 4.6: Western blot analysis of transiently expressed KSHV ORF22. HEK293 cells were transiently transfected with a number of ORF22 encoding plasmids. Cells were harvested after 48hours and supernatant, cell lysate (soluble and insoluble protein fraction) analysed by 12% SDS-PAGE. PVDF membranes were probed with the penta-his Mab.
1: pcDNA3.1-4.6gH
2: pcDNA3.1-5.1gH
3: pcDNA3.1-5.2gH
4: pcDNA3.1-7.11gH
5: Mock-transfected 293HEK cells
Type of sample is indicated at the bottom of each lane: Supernatant (S), insoluble (IN) or soluble (SOL).
Estimated molecular weight (in kDa) are shown in the left margins.

In summary, the expression of recombinant KSHV ORF22/gH and ORF47/gL was restricted in transient transfection systems. In particular for ORF22/gH the detection of low protein expression is limited to western blot procedures for the reasons mentioned earlier. Thus, higher amounts of protein are necessary. The establishment of stable cell lines expressing constitutively the protein of interest may increase the amount of protein synthesized and allow more distinct analyses of the two viral glycoproteins. The individual characterization of gH and gL proved to be more complex than initially anticipated. Consequently, experiments, examining a possible interaction between these two proteins were carried out at a later stage.

4.5.2 Stable 293HEK cell lines

In a transient transfection, cells represent a heterogeneous population, varying in their ability to produce recombinant protein. To produce a cell population where the majority express the protein, single cell clones have to be selected. The vector encodes the zeocin resistance gene allowing the growth of a positive transfectant in the presence of the drug zeocin. The minimum concentration required to kill HEK293 cells was determined in a kill curve experiment (data not shown). Transfected cells were routinely selected in the presence of 200-300μg/ml.

Mammalian cells were transfected as described before and incubated for 48 hours in complete growth medium. Cells were then split in a 1 in 10 ratio and transferred to complete medium containing 200μg/ml zeocin. After 10-14 days resistant cells were obtained, and aliquots of these stored in nitrogen. In addition cells were

cloned by limited dilution in 96 well plates (see section 2.2.6.5). Once clones were detected, cells were expanded and transferred to 12 well plates and subsequently tested for expression either by indirect immunofluorescence or by western blot. Six different plasmids, pcDNA3.1-4.2gL; pcDNA3.1-4.4; pcDNA3.1-5.3gL; pcDNA3.1-5.10gL; pcDNA3.1-4.6gH and pcDNA3.1-5.2gH were transfected and single cell clones obtained and analysed.

4.5.2.1 Initial screening of selected cells by PCR and indirect immunofluorescence

All cell lines established were initially screened by PCR for the presence of the insert. Cells were harvested and total DNA extracted using an extraction kit (Qiagen). A PCR mix was prepared containing the oligonucleotide primers described in chapter 3. Briefly, cell clones containing the gL construct should produce a 320bp fragment, and cell clones encoding the KSHV ORF22/gH a 778bp fragment. All cell lines tested were positive for their respective insert (data not shown).

To test for expression gL positive cell lines were seeded in cover slips and grown to 70% confluence, cells fixed in methanol/acetone and analysed for the c-myc tagged protein. Although these cells were cloned previously, the expression level was unexpectedly low in the majority of cell lines tested, with less than 15-20% of the cell population producing protein by IFA (data not shown). Some clones were found to be negative. The most promising cell lines were chosen and expanded for

more detailed analyses. Table 4.1 gives the names of the chosen stable cell lines, the plasmids transfected and their expression levels.

Name of cell line	Transfected plasmid	Expression level
293.4.2gL	pcDNA3.1-4.2gL	highest in IF, FACS,<30%
293.4.4gL	pcDNA3.1-4.4gL	relatively low, IF, <20%
293.5.3gL	pcDNA3.1-5.3gL	relatively low, IF, <20%
293.5.10gL	pcDNA3.1-5.10gL	high in IF, FACS, <30%
293.4.6gH	pcDNA3.1-4.6gH	relatively low, FACS, <20%
293.5.2gH	pcDNA3.1-5.2gH	relatively low, FACS, <20%

Table 4.1: Established 293HEK ORF22/gH or ORF47/gL stable cell lines. Cells were single cell cloned and tested by PCR as well as by a variety of methods, including indirect immunofluorescence; FACS analysis, western blot and blue cell assay.

Since recombinant ORF22/gH production could not be determined by IFA for the reasons described in chapter 3 and earlier in this chapter, PCR positive cells were expanded and tested by western blot. Results are discussed later in this chapter.

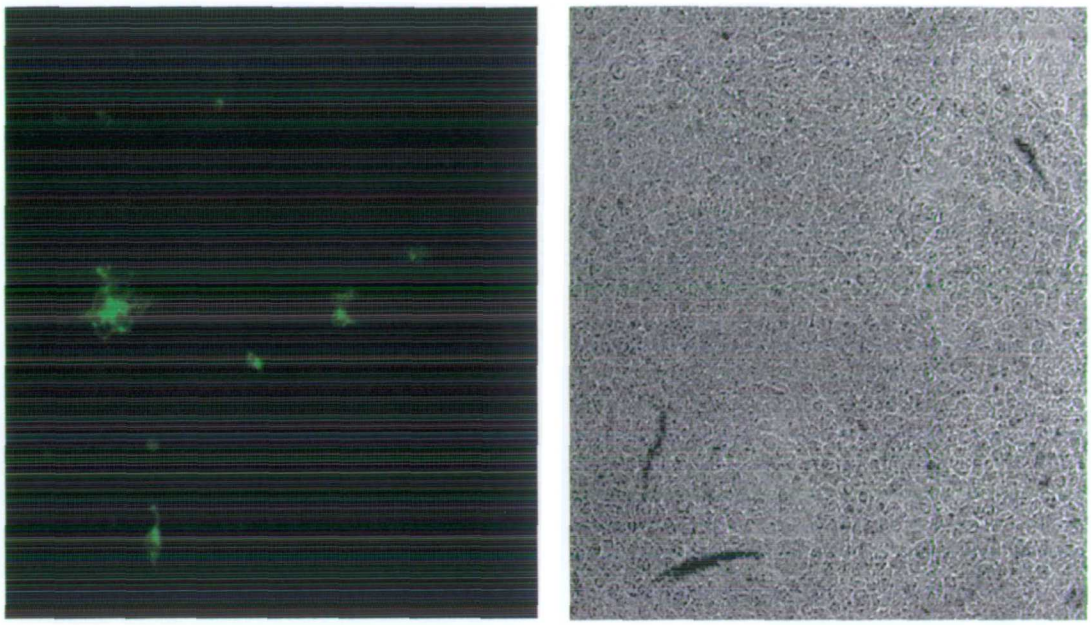
4.5.2.2 Analyses of stable cell lines

Cloned cell lines were expanded in selection media until a reasonable amount was available to test by a variety of procedures (Blue cell assay, indirect immunofluorescence, FACS, western blot). Cell growth was monitored and healthy cell cultures developed in the presence of zeocin.

4.5.2.2.a ORF47/gL expressing cell lines

Despite the selection process, the amount of protein produced in each cell line remained low. Blue cell assays were performed at each stage of the selection process to monitor protein expression. A constant decline in the number of blue cells could be observed (data not shown). However, a basic level of expression remained. Stable ORF47/gL cell lines were labeled with the monoclonal antibody 9E10 to measure the number of cells synthesizing protein. Expression could be observed, but at low levels. In figure 4.7A the fluorescence staining pattern, as well as the corresponding transmission picture demonstrate that approximately one or two cells out of every ten show fluorescence (10-20%). This result was confirmed by flow cytometry. Approximately $3x10^5$ cells were fixed, permeabilized and labeled with primary and secondary antibody in solution as described in chapter 2.2, section 7.7. Using a FACScan (Becton-Dickinson), 10^4 cells were counted and analysed for their fluorescence intensity; a histogram is shown in figure 4.7B. The level of fluorescence attributed to non-specific antibody interactions was determined by incubating the cells of interest with the FITC-conjugated secondary antibody alone (black line). Similarly, auto fluorescence of the cells themselves as well as the background noise of the

(A)



(B)

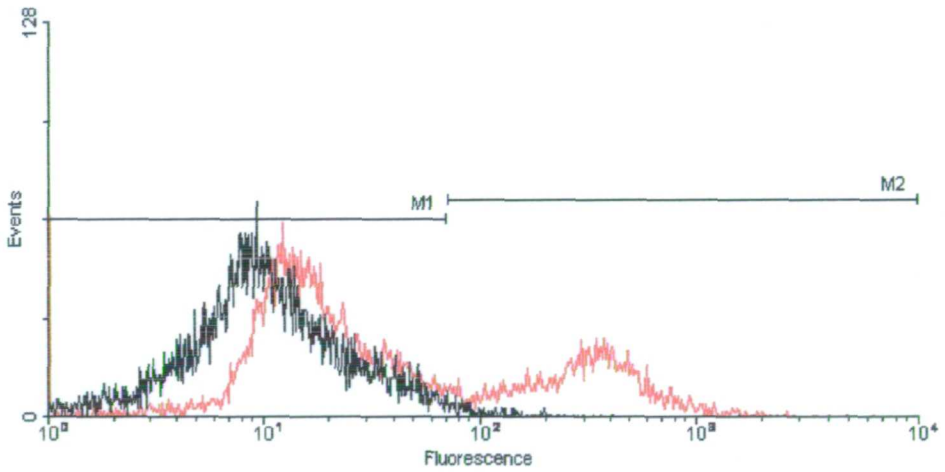


Figure 4.7: Evaluation of the expression level of gL expressing 293HEK stable cell lines. Cells were examined by IFA and flow cytometry as described in chapter 2.2. Cells were immunostained with Mab 9E10 and a goat anti-mouse FITC conjugated antibody. Pictures were taken with a confocal microscope. (A) Fluorescence and transmission image. (B) Flow cytometry histogram; red line indicates cells stained with the Mab 9E10 followed by FITC-conjugated secondary antibody, while the black line shows non-specific binding of the secondary antibody. Fluorescence intensity is shown on the x axis, and the relative number of cells is indicated on the y axis. This particular stable cell line (293.5.10gL) is typical of results obtained with all ORF47-expressing stable lines.

primary antibody were determined (data not shown). Background fluorescence intensity for the primary antibody (9E10), anti-mouse FITC conjugate and the cell line itself was on average 11.5 (Geo mean) and 8 (median) when 97% of the total cell population were gated (indicated by marker, M1 in figure). Fluorescence histograms show that two cell populations could be distinguished when negative control (cells incubated with FITC-conjugated secondary antibody only) and labeled cells (red line) were overlaid. One cell population having a similar fluorescence intensity as the negative control (black line), suggesting that these cells are unlikely to produce the recombinant protein and the signal is probably due to unspecific binding of the secondary antibody. In contrast, the second population showing a significant increase in fluorescence intensity, on average the Geometric mean was above 350 and the median mostly above 300 (marker, M2). In total approximately 10-20% of the total cell population seemed to express ORF47/gL in the cell lines tested.

Finally, to estimate the molecular weight of this protein and to examine whether the recombinant gL products were synthesized as soluble or insoluble proteins in mammalian cells, we collected cell lysates from a large number of cells ($2x10^7$) and monitored them by western blot. Cell lysates were harvested as described before, sonicated and stored at -20°C. To detect recombinant protein secreted into the culture medium, as described for HSV-1 gL, supernatant was also collected and concentrated using a Centricon-10 microconcentrator, for 2 hours at 6000 rpm and 4°C. All supernatant aliquots were negative for KSHV gL (data not shown).

The cell line 293.5.10gL synthesized a protein with an apparent molecular weight of 25-27kDa, that reacted with the monoclonal antibody 9E10 (see arrow in figure 4.8). Surprisingly, this protein could be predominantly found in the soluble protein fractions, rather than in the insoluble fraction as described for the gL homolog encoded by EBV (Pulford *et al.* 1995). The insoluble protein fractions all contained a faint protein band, migrating at the same size. This may be due to soluble protein contaminants.

As a control for non-specific binding, cell lysate from the parental 293HEK cell line was loaded in lanes 3 and 4. Only minor cross-reactivity could be detected, leading to the conclusion that the 25-27kDa protein corresponds to recombinant ORF47/gL. Taking into account the two glycosylation sites, this protein found in 293HEK cells may represent the fully-processed form of ORF47/gL. Additional experiments using an anti-ORF47/gL monoclonal antibody are essential to confirm these assumptions. However, at the time of writing such reagents do not exist. Further experiments confirmed that KSHV ORF47/gL can be predominantly detected in the soluble part of cell lysate (data not shown). The cell line described above was chosen for all further expression and localization studies of KSHV ORF47/gL.

4.5.2.2b Cell lines encoding ORF22/gH give a complex protein pattern

The ORF22/gH gene product synthesized in several cell lines was also analysed by western blot and flow cytometry as described above. Cell supernatant and lysates (soluble/insoluble) were collected and results are illustrated in figure 4.9.

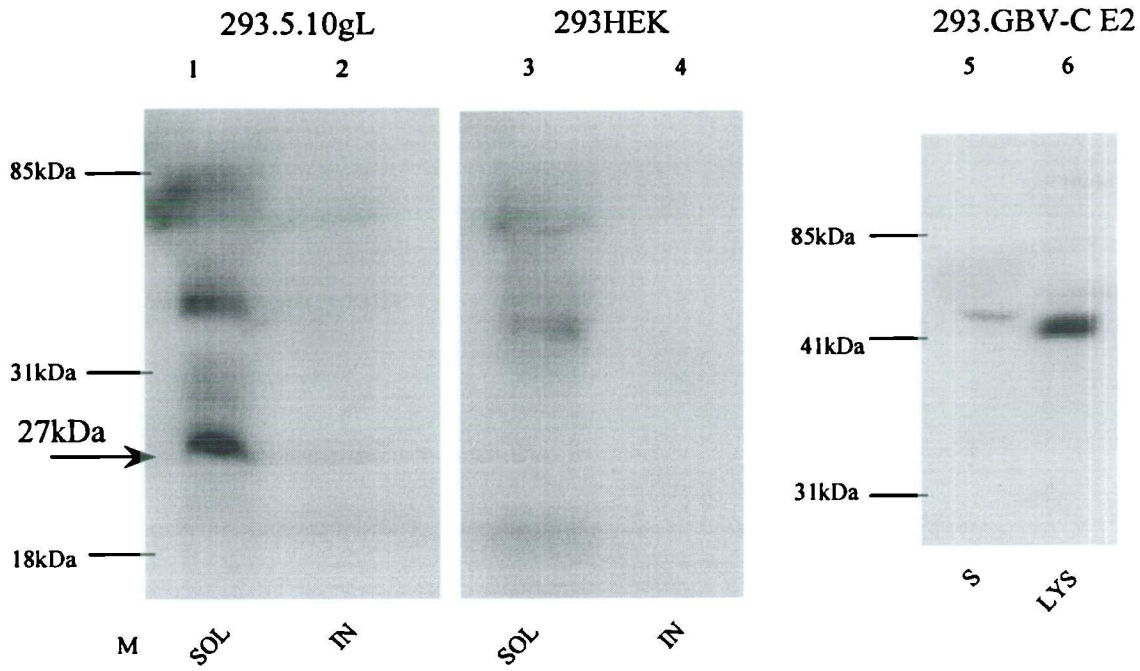


Figure 4.8: Western blot analysis of the gL expressing stable cell lines. Lysates were collected at different time points and loaded onto (12%) SDS-PAGE, electroblotted and examined with the Mab 9E10. Soluble (SOL) and insoluble (IN) protein fractions were analysed separately. Supernatant and cell lysate derived from the gL expressing cell line 293.5.10 are presented. The arrow indicates the 27kDa protein. As a negative control the parental cell line (293HEK) was also analysed by western blot as was the 293.GBV-C E2 stable cell line (positive control).
M: Molecular weight marker (Bio Rad)
Lane 1: 293.5.10gL, soluble protein fraction
Lane 2: 293.5.10gL, insoluble protein fraction
Lane 3: 293HEK, soluble protein fraction
Lane 4: 293HEK, insoluble protein fraction
Lane 5: 293.GBV-C E2, supernatant
Lane 6: 293.GBV-C E2, lysate (in Laemmli buffer)

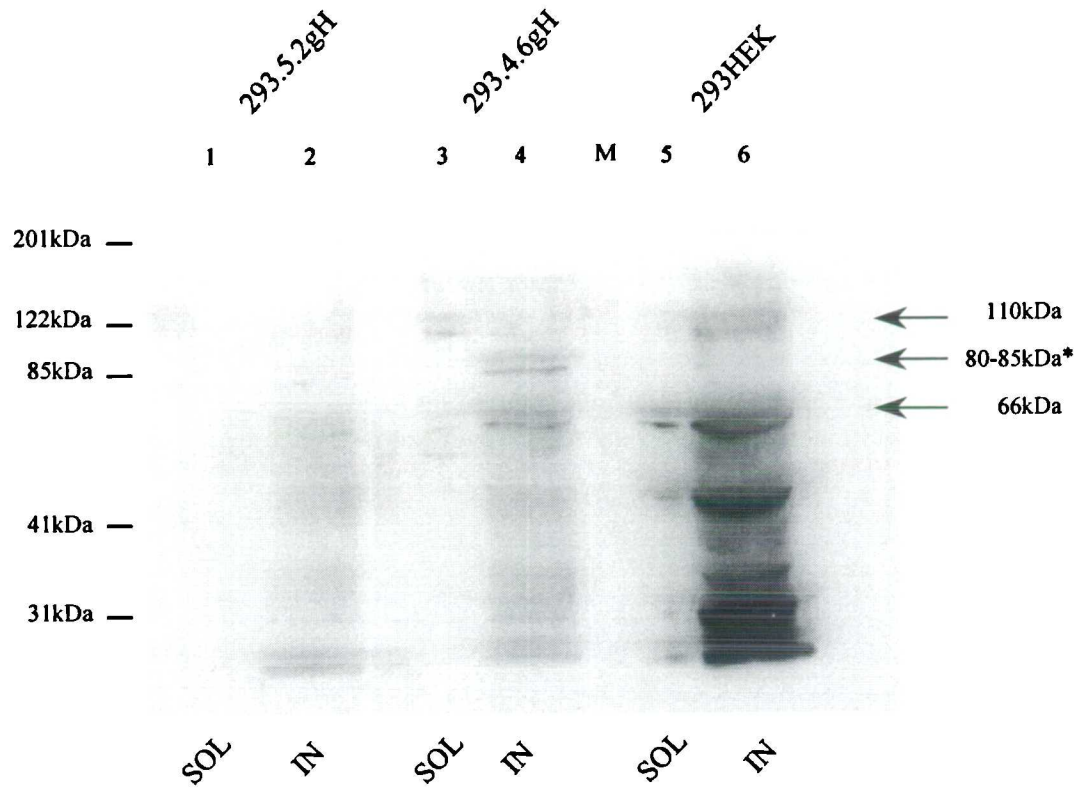


Figure 4.9: Western blot analysis of the gH expressing stable cell lines. Lysates were collected at different time points and loaded onto (7%) SDS-PAGE, electroblotted and examined with the penta-his monoclonal antibody. Soluble and insoluble protein fractions were analysed separately. Arrow indicates the putative KSHV gH protein mentioned in the text.
Lane 1: 293.5.2gH, soluble (SOL) protein fraction
Lane 2: 293.5.2gH, insoluble (IN) protein fraction
Lane 3: 293.4.6gH, soluble protein fraction
Lane 4: 293.4.6gH, insoluble protein fraction
M: Molecular Weight marker (Bio Rad)
Lane 5: 293HEK, soluble protein fraction
Lane 6: 293HEK, insoluble protein fraction
*The 80-85kDa protein band can be seen weakly in lane 4. For a better visualization see figure 4.13, lanes 2 and 4.

Once more, protein expression was low and detailed studies of the recombinant protein were complicated. Both cell lines, 293.4.6gH and 293.5.2gH (illustrated in lane 1, 2, 3, 4), synthesized the earlier described 66kDa protein and a weakly reacting additional protein of 80-85kDa that was limited to the insoluble protein fraction (for a better result see figure 4.13) and was absent in the negative control (lanes 5 and 6). Furthermore, a band with less electrophoretic mobility (app. 110kDa) was also detected in the soluble protein fraction of cells harboring the ORF22/gH from patient 4 (lane 3). However, several proteins produced by the parental, non-transfected, 293HEK cells, also reacted with the histidine specific antibody, making the result difficult to interpret. With respect to the negative control lysate, the 85kDa protein, synthesized by the selected cell lines may correspond to KSHV ORF22/H, but an exact identification would need confirmation by an anti-ORF22/gH antibody.

FACS analyses of cell lines encoding ORF22/gH demonstrate that less than 20% of the total cell population had elevated fluorescence levels, distinguishable from the negative control (data not shown). A control cell line expressing the reporter protein his-β-galactosidase was used to determine the binding activity of the penta-his monoclonal antibody in this particular assay. The control plasmid pcDNA3.1-his-β-galactosidase was transfected in duplicate to test for protein expression by two independent methods, flow cytometry (detecting the histidine tag) and X-Gal staining, detecting the β-galactosidase. Surprisingly, FACS analyses and histochemical staining procedures of the same transfected cells did not correspond, blue cells could be detected routinely, but flow cytometric

methods showed no significant increase in fluorescence (data not shown). Thus, any results obtained with the penta-his antibody were interpreted with care. However, since the cell line 293.4.6gH expressed the highest amount of the 85kDa protein, under the circumstances described, it was chosen for further studies.

To conclude, these experiments show that, despite single cell cloning, the level of expression could not be increased to 100%. Flow cytometry experiments estimated that only 20-30% of cells produce recombinant protein. For examination of biochemical properties, such as glycosylation pattern or possible in vitro complex formation between ORF22/gH and ORF47/gL, proteins need to be available at higher amounts. A final approach was made to manipulate protein expression. Sodium butyrate was added to the culture medium of stable cell lines and its influence on expression level monitored. For intracellular distribution studies these stable cell lines were examined by confocal microscopy. Results are detailed later chapter 5.

4.5.2.3 Sodium butyrate induction of stably selected cell lines

Some promoters are susceptible to chemical stimuli and can be influenced in their ability to regulate gene expression, e.g. herpesviral lytic expression can be induced by the addition of sodium butyrate (see chapter 1). Sodium butyrate has been demonstrated to affect transcription regulation and enhance gene expression in eukaryotic cells (Kruh, 1982). Studies showed that butyrate significantly enhances expression of genes controlled by either the CMV-IE promoter/enhancer or the

SV40 promoter (Condreay *et al.* 1999). The addition of butyrate to the growth medium markedly elevates gene expression of a recombinant protein in several cell lines (CHO; HeLa; Cos-7; HEK293) when under the control of the respective promoter (Palermo *et al.* 1991; Condreay *et al.* 1999). Butyrate has a variety of effects on different cell types, the underlying mechanism behind this ability to stimulate transcription is thought to be mediated by inhibition of the enzyme histone deacetylase (Kruh, 1982).

To increase the expression level of the established stable cell lines a range (1, 2, 5 and 10mM) of butyrate concentrations were tested. The chemical was added to the growth medium and cells monitored for detachment. Concentrations above 10mM markedly inhibited cell growth; lower concentrations were tolerated though cell growth was slowed down (data not shown). For expression purposes cells have to be in S-phase as the CMV-based mammalian expression vector are cell cycle and serum dependent (Brightwell *et al.* 1997). Thus, 2-8mM of the chemical were used in the following induction studies.

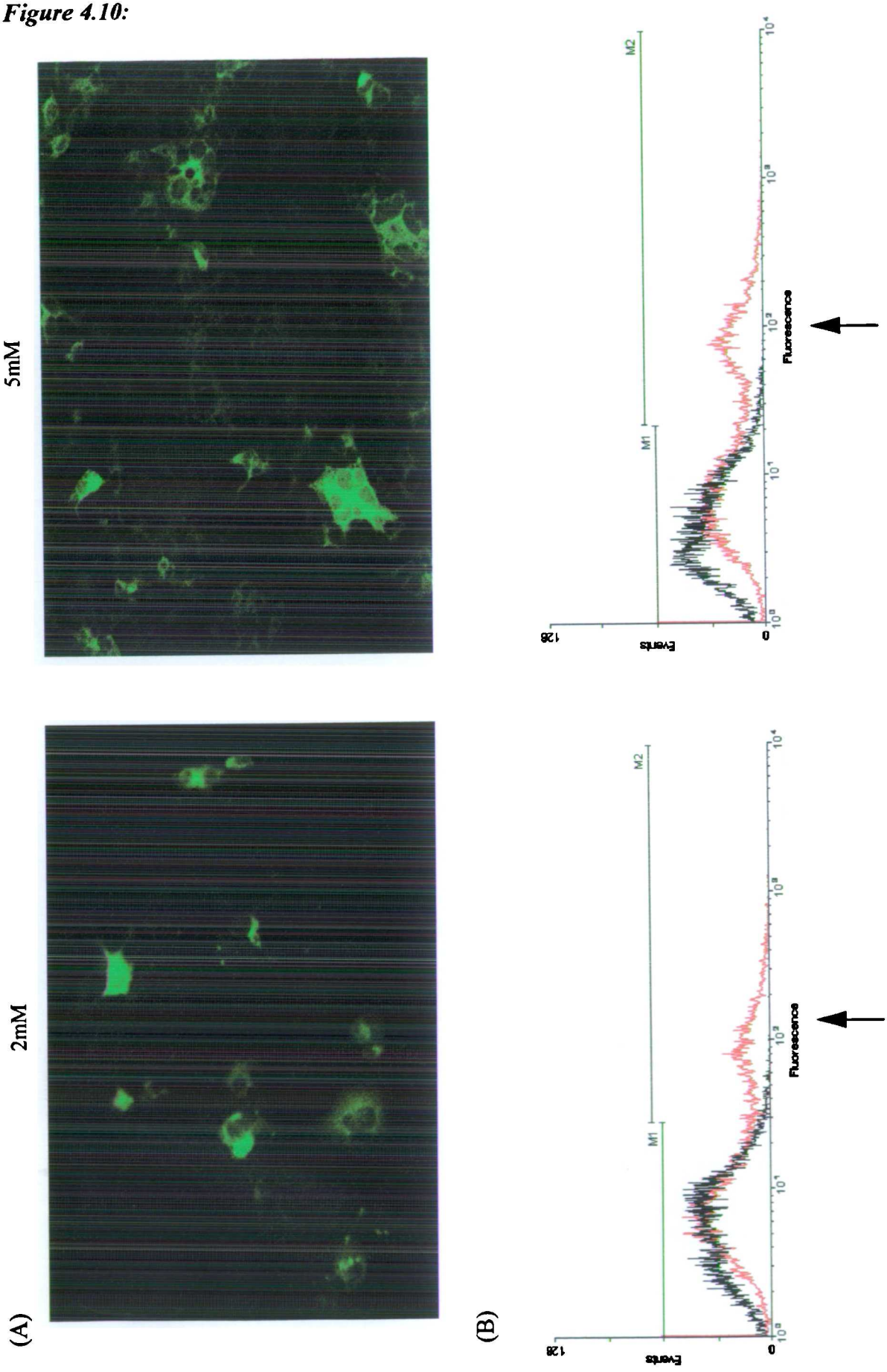
4.5.2.3a Influence of chemical stimuli on protein expression analysed by IFA and flow cytometry

Chemical induction of protein expression was focused on ORF47/gL-expressing cell lines, since studies using the penta-his antibody for these specific detection assays were inconsistent. Experiments were performed in duplicate, cells being seeded into cover slips for IFA and into 60mm dishes for flow cytometry. Following 24 hours incubation culture medium was removed and replaced by

fresh medium supplemented with different concentrations of sodium butyrate (2mM, 5mM). Cells were incubated for a further 16 hours. Harvest, fixation and subsequent staining was performed as set out in chapter 2.2, section 7.6. Recombinant protein expression was considerably increased in the presence of butyrate. Both, confocal micrographs and the corresponding flow cytometry histogram obtained following induction with n-butyrate are shown in figure 4.10. The concentration of n-butyrate is shown above each picture. In comparison to untreated cells (compare figure 4.10 to figure 4.7) it is clear that butyrate has a significant influence on ORF47/GL expression. Cells incubated in the presence of butyrate (figure 4.10) exhibit an intense green color on IFA. This observation is also reflected in the corresponding FACS histograms (panel B). Two populations can be distinguished throughout, but the fluorescence intensity of the right curve (red), representing gL-expressing cells, is increased when butyrate is added, in particular where both curves overlap (see arrow).

Figure 4.10 (next page): Sodium butyrate induction of gL encoding 293 stable cell lines. Cells were incubated over night with sodium butyrate; concentrations are indicated above each IFA image. Protein expression was analysed by (A) IFA and (B) flow cytometry as detailed in Material and Methods. Following induction cells were fixed or harvested and stained with the Mab 9E10. Bound antibody was detected with FITC-conjugated goat anti-mouse antibody. Red line: cells labeled with primary and secondary antibody; black line: cells labeled with secondary antibody only. The arrow highlights differences in expression level.

Figure 4.10:



This data indicates an increase in the number of cells synthesizing ORF47/gL when under the influence of n-butyrate. To summarize the effect of butyrate on recombinant expression, the percentage of cells exhibiting fluorescence is plotted against the concentration of butyrate added, 2, 5 and 10mM respectively (figure 4.11). The influence of 10mM butyrate was also assessed by flow cytometry but the effect was reversed. A further increase in expression, in comparison to 5mM, could not be noticed. It is probable, that at this high concentration, cell growth was inhibited and as a consequence the amount of protein reduced.

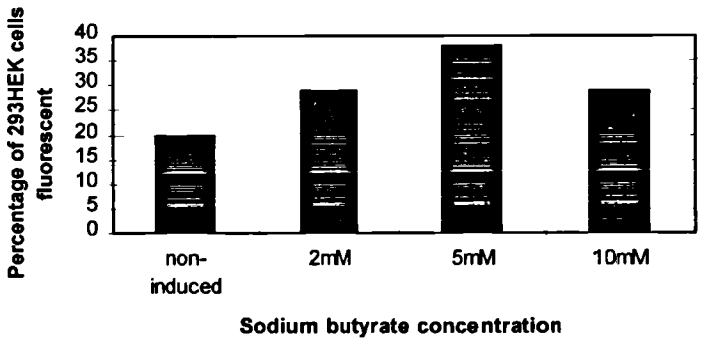


Figure 4.11: Influence of sodium butyrate on recombinant protein expression in the stable cell line 293.5.10gL. Cells were incubated over night in the presence of different concentrations of sodium butyrate. Following fixation, cells were examined by flow cytometry as detailed before. Histograms were prepared using the computer software WinMDI. Approximately 97% of the total negative cell population was gated and the amount of cells exhibiting gL expression determined. The mean fluorescence intensity ranged from 250 to 300.

This data suggests that stable cell lines contain the appropriate insert but the level of ORF47/gL expression is low despite single cell cloning. To complete the expression studies in HEK293 cells lysates obtained from butyrate-treated cells were examined by western blot.

4.5.2.3b Final western Blot showing recombinant ORF47/gL and ORF22/gH gene products

Encouraged by these elevated expression levels, all stable cell lines were re-evaluated in western blot procedures after being treated with 8mM sodium butyrate. Cells were harvested as before, soluble and insoluble protein fractions separated and approximately 10^6 cells loaded per well of a 7% or 12% SDS-PAGE. Proteins were transferred to PVDF membrane and probed with either 9E10 or penta-his monoclonal antibody.

Figure 4.12 demonstrates the recombinant ORF47/gL produced after butyrate induction of the stable cell lines 293.4.2gL and 293.5.10gL. Several bands reacted with the 9E10 Mab. A faint and diffuse band, migrating at 27kDa could be visualized in the soluble part of 293 cells transfected with the 5.10 plasmid, in contrast to the 293.4.2gL cell line, where this protein was absent. This protein has been described before (section 4.5.2.2.a) and most likely corresponds to the mature form of ORF47/gL. The diffuse pattern also indicates possibly glycosylation. Other experiments, using different concentrations of butyrate and analysing expression by western blot, confirmed data obtained earlier by flow cytometry, in that the expression level is directly dependent on the concentration

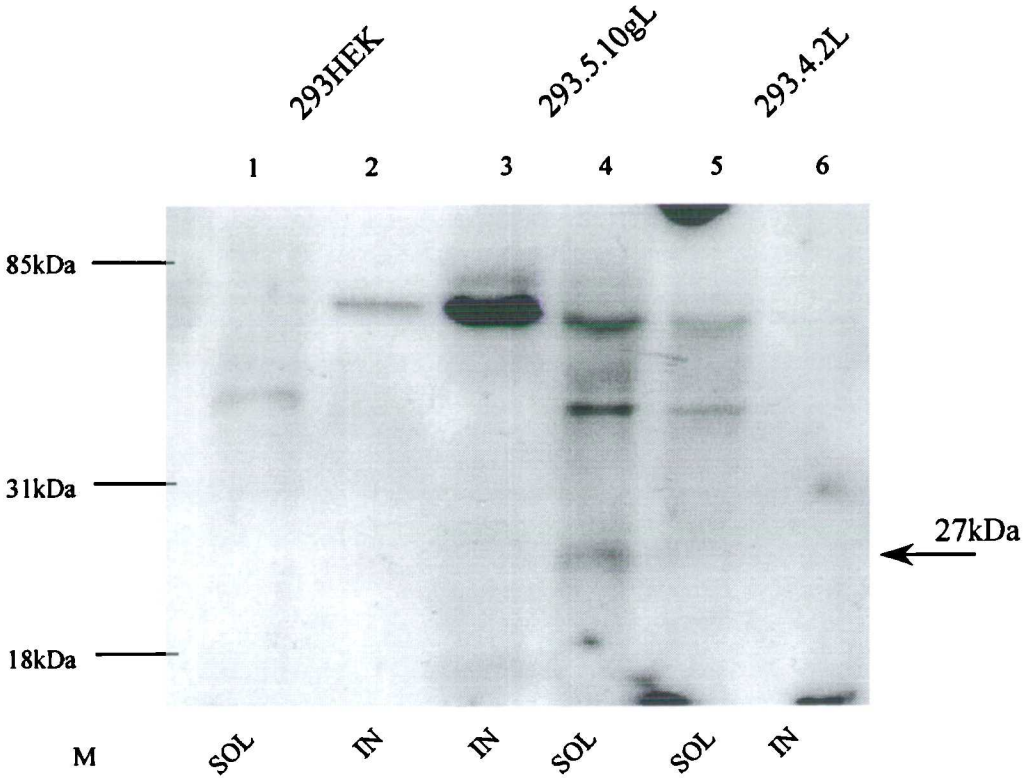


Figure 4.12: Re-evaluation of gL expressing 293 stable cell lines following sodium butyrate induction. Each cell line was induced with 8mM (f.c.) sodium butyrate over night. Cell lysate was prepared as described before and approximately 10^6 cells were loaded per well of a 12% denaturing polyacrylamide gel. Proteins were transferred to PVDF membrane and labeled with the monoclonal antibody 9E10. Arrow indicates KSHV ORF47/gL gene product.
M: Molecular Weight marker (Bio Rad)
Lane 1: 293HEK, soluble protein fraction
Lane 2: 293HEK, insoluble protein fraction
Lane 3: 293.5.10gL insoluble protein fraction
Lane 4: 293.5.10gL soluble protein fraction
Lane 5: 293.4.2gL soluble protein fraction
Lane 6: 293.4.2gL insoluble protein fraction

of butyrate added to the culture (data not shown). The western blot shown in figure 4.12 represents the highest amount of recombinant protein we could achieve by using a small amount of cells and inducing them over night.

Several other slow migrating proteins could also be visualized in the non-transfected cell line (>41kDa; lanes 1 and 2), suggesting cross-reactivity.

However, no band was present at 27kDa within the parental cell line, indicating that 293HEK cells are able to fully process ORF47/gL as a soluble protein.

Cell lysates from ORF22/gH-encoding cell lines were also subjected to SDS-PAGE and analysed by western blot using the penta-his monoclonal antibody. As hoped, a positive result was also obtained for those cell lines, as demonstrated in figure 4.13. Several protein species could be identified, ranging from approximately 80-180kDa. The 80-85kDa band was present predominantly in the insoluble protein fraction, together with a protein migrating more slowly (100-110kDa). This 80-85kDa band had already been detected in transient transfections and may correspond to an alternatively processed form of ORF22/gH (the predicted mass, without sugar, is approximately 79kDa). The second protein reacting with the monoclonal antibody is likely to represent the mature form of ORF22/gH. To further confirm this statement studies examining the glycosylation pattern of ORF22/gH would have to be performed. The soluble part of the cell lysates also contained the 80-85kDa protein, although at a lower level. Within lysates derived from non-transfected 293HEK cells the proteins described above were absent (lane 5 and 6). Lane 7 shows the antibodies activity towards the transiently expressed control protein, his-βgalactosidase. Overall, the expression

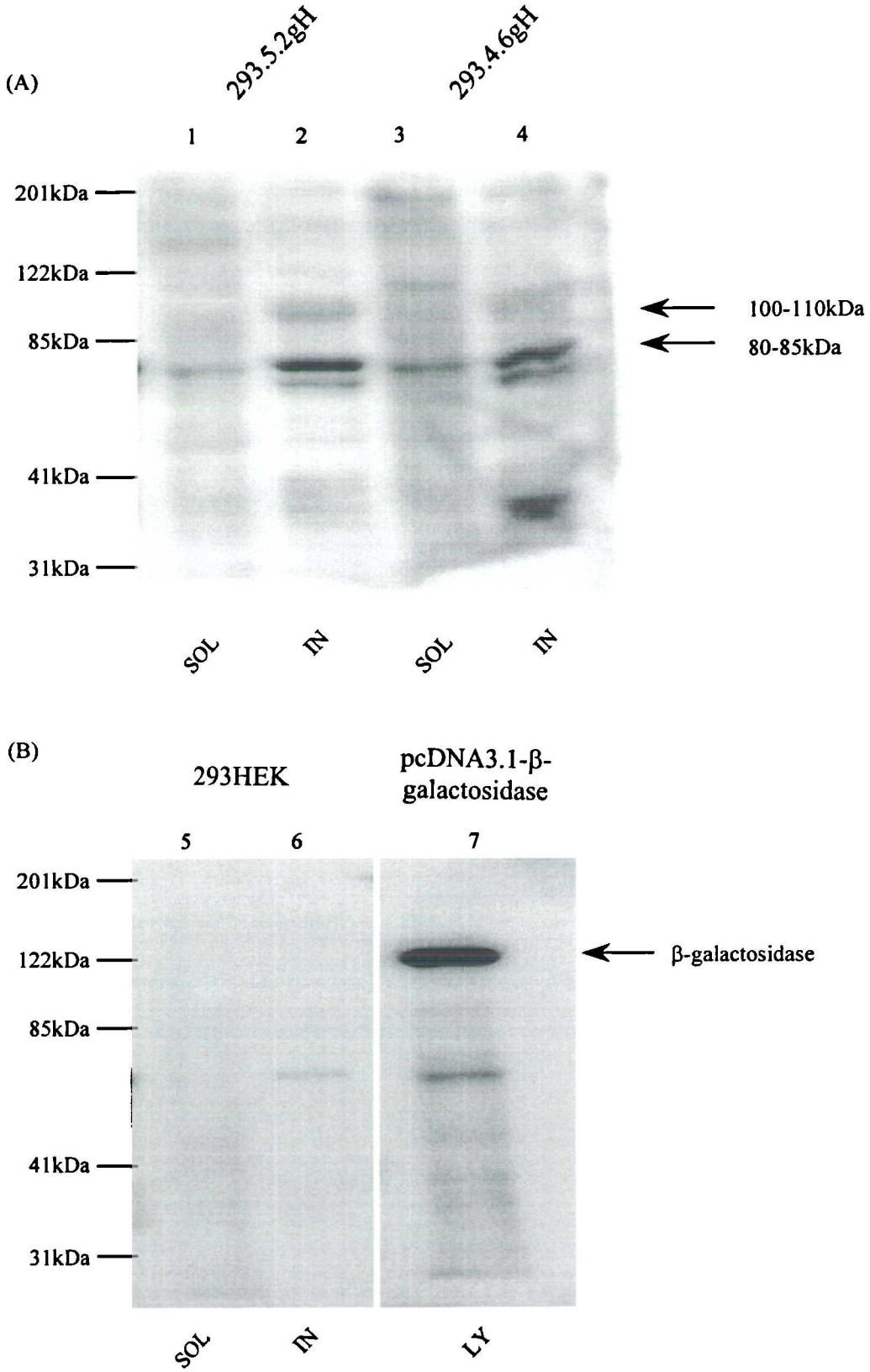
pattern is consistent with the proteins seen in previous transfection experiments and supports the conclusion that 293HEK cells produce several forms of ORF22/gH as insoluble protein.

To conclude, using sodium butyrate, detection of the two glycoproteins could be achieved and their approximate molecular weights estimated. Further investigations using monoclonal antibodies specific for these KSHV open reading frames will be essential to rule out possible cross reactivity of the monoclonal antibodies 9E10 and (especially) penta-his.

The expression of ORF47/gL and ORF22/gH seems to be complex, a cell line expressing higher levels of protein without chemical induction would be desirable as the influence of sodium butyrate on the biochemical properties of the proteins of interest is not known.

Figure 4.13 (next page): Re-evaluation of gH expressing 293 stable cell lines following sodium butyrate induction. Each cell line was induced with 8mM (f.c.) sodium butyrate over night. Cell lysate was prepared as described before and approximately 10^6 cells were loaded per well of a 7% denaturing polyacrylamide gel. Proteins were transferred to PVDF membrane and labeled with the penta-his antibody. Arrow indicates the putative KSHV ORF22 gene product. Molecular weight marker is indicated on the left. (SOL) soluble; (IN) insoluble, (LY) lysate.
(A) Lane 1: 293.5.2gH soluble protein fraction (SOL)
Lane 2: 293.5.2gH insoluble protein fraction (IN)
Lane 3: 293.4.6gH soluble protein fraction (SOL)
Lane 4: 293.4.6gH insoluble protein fraction (IN)
(B) Lane 5: 293HEK parental cell line, soluble protein fraction
Lane 6: 293HEK parental cell line, insoluble protein fraction
Lane 7: 293HEK, transiently transfected with pcDNA3.1-β-galactosidase, in Laemmli buffer.

Figure 4.13: Legend see page 217.



4.5.3 The Cos7 cell line

4.5.3.1 Recombinant expression in Cos7 cells

As described earlier in this chapter, Cos7 cells encode the SV40 large T-antigen and therefore support episomal replication of expression vectors containing an SV40 origin of replication, such as pcDNA3.1. Transfected cells may therefore contain several copies of the vector, leading to a theoretical increase in the synthesis of recombinant protein per cell. We therefore chose to determine expression of gH and gL in this second cell line.

Optimal transfection conditions were determined using the control plasmid pcDNA-his-β-galactosidase and the transfection reagent effectene as described above. Approximately 1μg DNA and an effectene ratio of 1 in 25 resulted in the highest level of expression as judged by blue cell staining and western blot (data not shown). To establish the expression level of the plasmids indirect IFA and flow cytometry analyses were performed. Both methods indicated a low level of transient expression by IFA and made western blot procedures unnecessary (see below). Several stable Cos7 cell lines were established and tested for the presence of recombinant ORF22/gH and ORF47/gL. Although the respective sequences could be detected by PCR for each cell line, the level of expression was low as measured by IFA and FACS analyses (data not shown). In conclusion, the construction of cell lines constitutively expressing these KSHV ORFs appears to be a rare event (and rather time consuming). Subsequent experiments therefore focused on a transient transfection system in combination with sodium butyrate induction.

4.5.3.1a Cos7 cells show no improved expression pattern

To compare the expression with 293 cells, the same plasmids, including 4.2gL, 5.10gL and each together with 4.6gH or 5.2gH, were transfected into Cos7 cells. The following morning cell culture medium was replaced as a significant amount of cell detachment could be observed. After an additional 24 hours, cells were fixed in methanol/acetone and indirect immunofluorescence performed with the monoclonal antibody 9E10. A FITC image and the corresponding transmission image showing gL transfected Cos7 cells are shown in figure 4.14A.

A similar result was obtained with gH/gL co transfections (data not shown). The number of fluorescent cells was low, regardless of which plasmid was transfected. Interestingly, the cells which did express the protein had a "damaged" phenotype when compared to mock transfected cells (see transmission image, figure 4.14C). This observation suggests that the expression of ORF47/GL could effect cell growth, possibly forcing the host cell to inhibit recombinant protein production to survive. Additional flow cytometry analyses confirmed the IFA results and allowed us to quantify the number of expressing cells. Around 10-15% of the total cell population could be detected, with a mean fluorescence intensity in the range of 100 to 183, and background values between 30 and 40. Overall, the expression pattern of ORF47/GL in Cos7 cells shows no remarkable difference from transiently or stably transfected 293 cells. In addition several attempts were made to demonstrate the two glycoproteins in immunoblots (data not shown). As expected from IFA studies and FACS for ORF47/gL, the amount of protein was

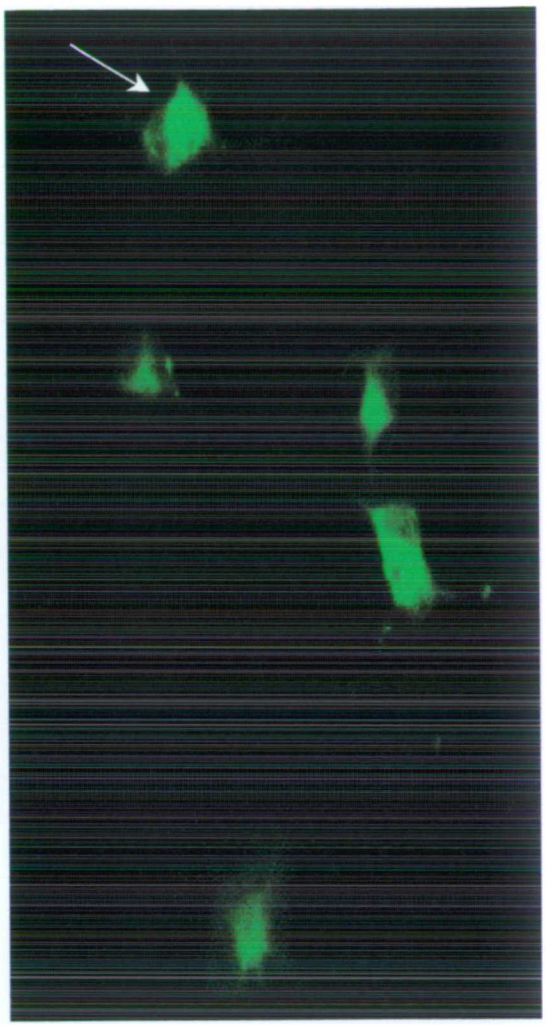
insufficient to be detected by this method. Unfortunately, there was no alternative method to detect the synthesis of ORF22/gH when singly transfected.

Further studies therefore concentrated on transient transfections combined with sodium butyrate induction and confocal microscopy of cells expressing gL alone or together with gH.

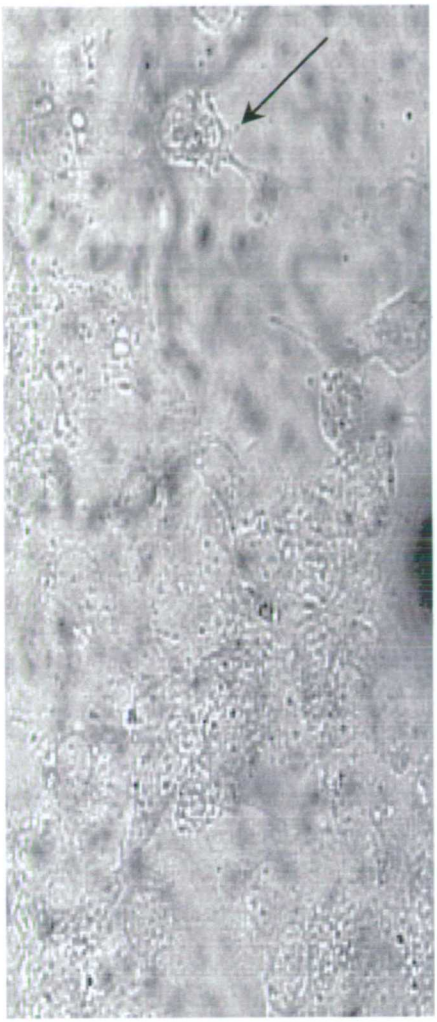
Figure 4.14 (next page): Immunofluorescence analyses of gL expression in Cos7 cells after transient transfection. Cells were transfected in cover slips and fixed in methanol/acetone 48 hours post-transfection. Fixed cells were then reacted with the Mab 9E10, bound antibody was detected by reaction with FITC-conjugated goat anti-mouse antibody. Cells were subsequently viewed by confocal microscopy. Both, the FITC image (A) and the corresponding transmission images (B) are shown. Arrows indicate corresponding cells. In panel C mock-transfected Cos7 cells are shown. Cos7 cells transfected with pcDNA3.1-4.2gL represent data obtained with other plasmids.

Figure 4.14:

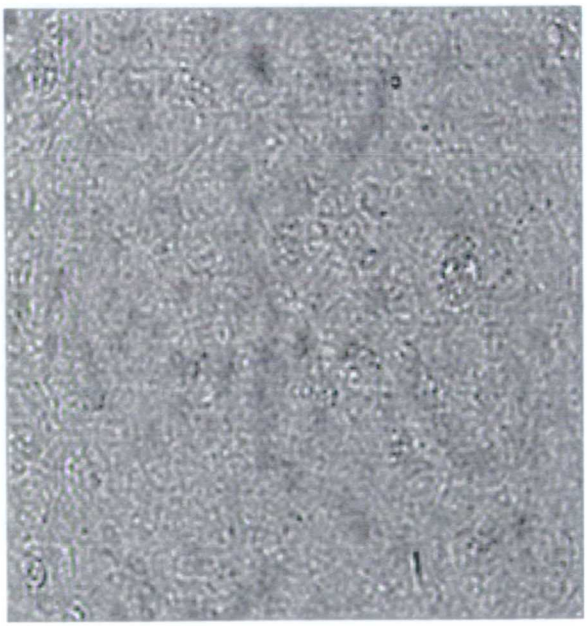
(A)



(B)



(C)



4.5.3.2 Sodium butyrate, an inducer of recombinant protein expression

Although SV40 large T antigen positive cells were expected to produce higher amounts of recombinant protein, our experiments revealed the opposite for KSHV ORF22/gH and ORF47/gL. Protein levels remained low, even after clonal cell lines had been obtained. As a consequence a transient expression system was developed where transfectants were chemically induced shortly after transfection, with the aim to enhance protein expression. Previous studies using the control plasmid demonstrated that the transfection efficiency is satisfactory (approximately 50%), the addition of sodium butyrate after the transfection may result in an increase in expression.

Cells were transfected in duplicate with different gH and gL encoding plasmids. After 16 hours supernatant was removed and fresh growth medium, supplemented with 5mM sodium butyrate, added and the cells incubated for a further 24 hours. Supernatants were collected and cells analysed by western blot and flow cytometry. Higher amounts of butyrate could not be used because of its inhibition and toxicity for cell growth (data not shown).

4.5.3.2a The ORF47/gL gene product expressed in Cos7 cells

Cell supernatants, soluble and insoluble protein fractions were separated on a 12% SDS-PAGE, transferred to PVDF membrane and probed with the monoclonal antibody 9E10 (Figure 4.15).

A prominent band was present in the soluble protein fraction of gL-transfected cells (lanes 1 and 3), but absent in the negative control. The protein was 27kDa in size, concomitant with the pattern previously seen in 293 cells. This protein most

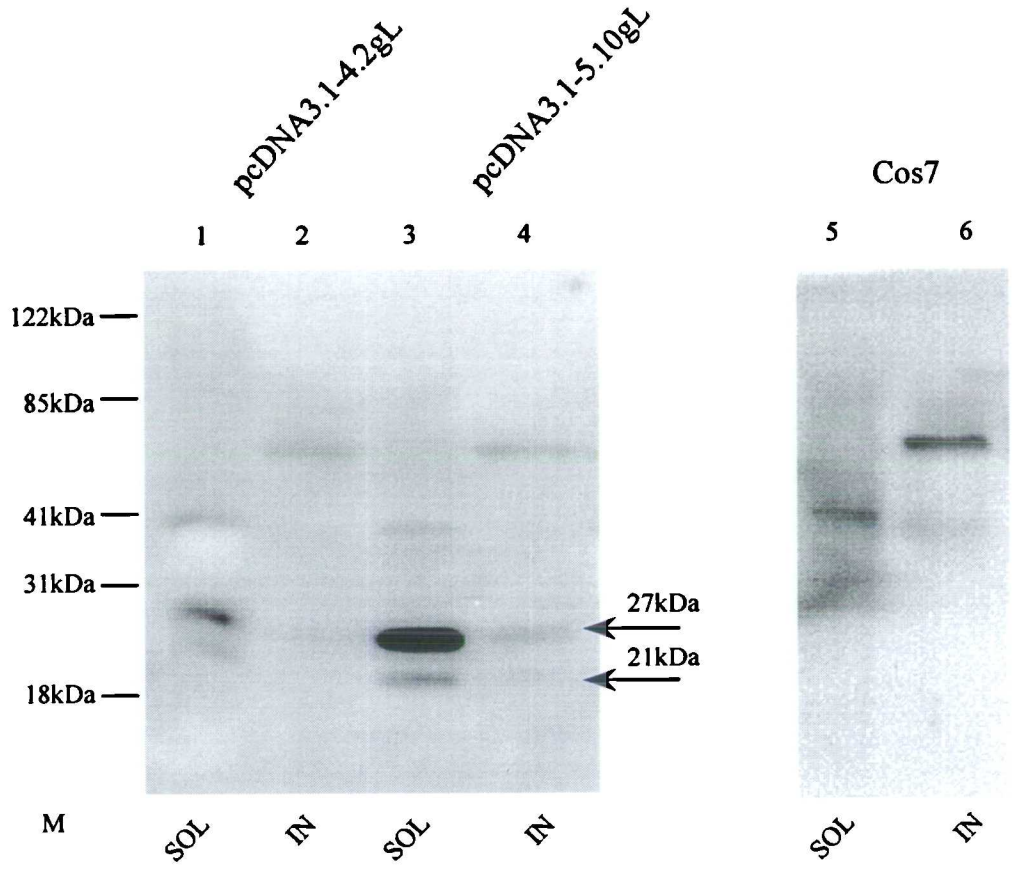


Figure 4.15: Proteins expression in Cos7 cells after transient transfection combined with sodium butyrate induction. Cells were transfected with KSHV ORF47 plasmids, induced after 24 hours and incubated for an additional 24 hours.
Supernatant was collected and cells harvested as described before. Protein extracts were resolved on 12% denaturing polyacrylamide gel, transferred to PVDF membrane and probed with the Mab 9E10. Arrows indicate KSHV ORF47 gene product.
Lane 1: pcDNA3.1-4.2gL, soluble protein fraction
Lane 2: pcDNA3.1-4.2gL, insoluble protein fraction
Lane 3: pcDNA3.1-5.10gL, soluble protein fraction
Lane 4: pcDNA3.1-5.10gL. insoluble protein fraction
Lane 5: Cos7 cells, mock-transfected, soluble protein fraction
Lane 6: Cos7 cells; mock transfected, insoluble protein fraction
Molecular weight is indicated at the left side.

likely corresponds to a processed form of KSHV ORF47/gL. Interestingly, a second protein not seen in 293 stable cell lines, with an approximate size of 21kDa was also detected in lanes 1 and 3. This protein reacted only weakly with the antibody and may represent an immature form of ORF47/gL. As seen with 293 stable cell lines, cells transfected with the pcDNA3.1-5.10gL plasmid appear to produce gL more efficiently than cells harboring the 4.2gL plasmid. Reasons for this difference remain to be determined.

Overall, these data suggest that Cos7 cells are able to produce ORF47/gL and to perform post-translational modifications for most of the protein synthesized, though less efficiently than stable 293HEK cell lines.

4.5.3.2b The ORF22/gH gene product expressed in Cos7 cells

Cos7 cells were also transfected with ORF22/gH expressing plasmids and cell lysates prepared following sodium butyrate induction. The western blot is shown in figure 4.16. The gH protein profile contained a major band with an apparent mass of approximately 80-85kDa, predominantly found in the insoluble protein fraction. Furthermore, a second band, 110kDa in size, that reacted weakly with the penta-his antibody could also be detected. As described for 293 cells, this band may represent a processed form of ORF22/gH, whereas the 80-85kDa most likely corresponds to the pre-protein form. Both plasmids exhibited a similar expression level, as judged by the band intensity. Mock-transfected lysate did not produce a protein at this size that reacted with the monoclonal antibody (data not shown).

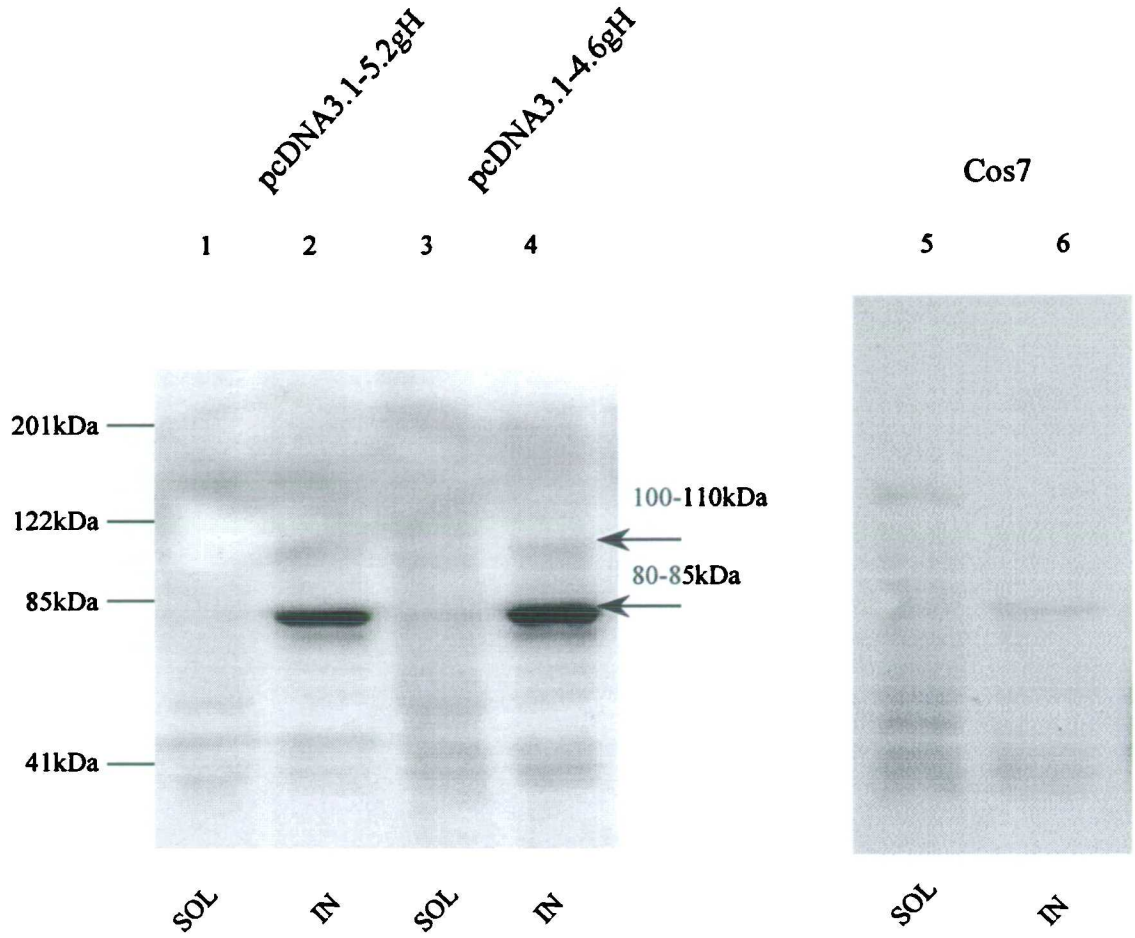


Figure 4.16: Proteins expression in Cos7 cells after transient transfection combined with sodium butyrate induction. Cells were transfected with KSHV ORF22 plasmids, induced after 24 hours and incubated for an additional 24 hours.
Supernatant was collected and cells harvested as described before. Protein extracts were resolved on 7% denaturing polyacrylamide gel, transferred to PVDF membrane and probed with the penta-his Mab. Putative proteins are indicated by an arrow.
Lane 1: pcDNA3.1-5.2gH, soluble protein fraction
Lane 2: pcDNA3.1-5.2gH, insoluble protein fraction
Lane 3: pcDNA3.1-4.6gH, soluble protein fraction
Lane 4: pcDNA3.1-4.6gH, insoluble protein fraction
Lane 5: Cos7 cells, mock-transfected, soluble protein fraction
Lane 6: Cos7 cells; mock transfected, insoluble protein fraction
Mock-transfected Cos7 cells were analysed separately, showing no band at the same position as transfected cells (data not shown).

4.5.3.2c Co- expression of both gH and gL

Cellular lysates obtained from co transfections were also examined for protein expression and results are shown in figure 4.17. The membrane was first probed with the penta-his antibody (A), subsequently stripped according to the manufacturers instructions, and re-probed by 9E10 (B).

Interestingly, co transfected cells produced a similar protein pattern as seen for individual transfected cells. ORF22/gH was expressed predominantly as an insoluble 80-85kDa protein (lane 2) and no increase of processed ORF22/gH could be observed. Also the co expression did not result in larger amounts of soluble protein. In contrast, ORF47/gL was fully processed and only the 27kDa glycoprotein was present in the soluble protein fraction (panel B, lane 1). The immature (21kDa) form of ORF47/gL seemed to have disappeared; no protein similar in size reacted with the 9E10 monoclonal antibody. Whether this is caused by the presence of ORF22/gH remains to be determined, but it would be hard to prove as the amount observed in single transfections was already low.

In summary, this chapter details a series of experiments which eventually led to a reliable system whereby both proteins ORF22/gH and ORF47/gL could be successfully expressed in both 293HEK and Cos7 cells. The results obtained are more or less similar and indicate that ORF47/gL exists mostly in its processed form, whereas ORF22/gH is also produced as a pre-protein. In the following chapter localization studies focused on the distribution pattern of ORF47/gL with and without ORF22/gH.

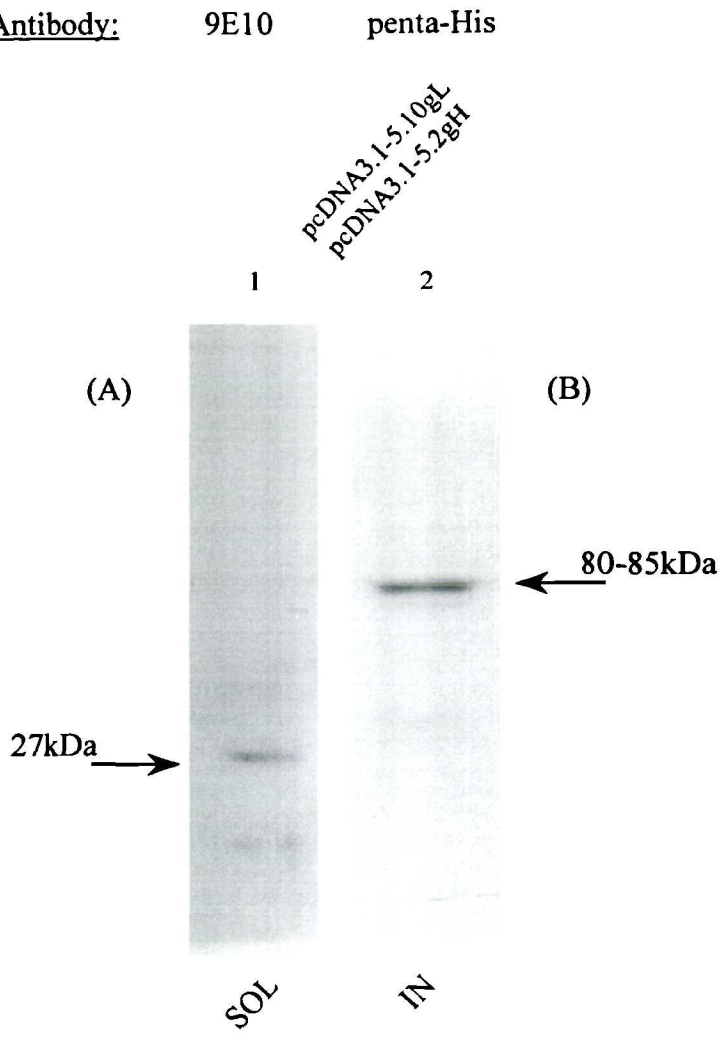


Figure 4.17: Transient co expression of KSHV ORF47/gL and ORF22/gH in Cos7 cells following sodium butyrate induction. Cells were co-transfected with both plasmids, induced after 24 hours and incubated for an additional 24 hours.
Cells were harvested as described before and protein extracts resolved on (A) 12 and (B) 7% denaturing polyacrylamide gel, transferred to PVDF membrane and probed with the 9E10 Mab or the penta-his Mab. (SOL) soluble; (IN) insoluble.
Lane 1: pcDNA3.1-5.10gL/pcDNA3.1-5.2gH; soluble protein fraction
Lane 2: pcDNA3.1-5.10gL/pcDNA3.1-5.2gH; insoluble protein fraction
Arrows indicate the KSHV ORF22 and ORF47 gene products.

4.6 Summary and Discussion

Intracellular transport and processing of a variety of herpesviral gH and gL homologs have been analysed, mostly in transient expression systems, including recombinant vaccinia virus in mammalian cells or baculovirus in insect cells (Browne *et al.* 1993; Dubin and Jiang, 1995; Duus *et al.* 1995; Duus and Grose, 1996; Foa-Tomasi *et al.* 1991; Forrester *et al.* 1991; Gompels and Minson, 1989; Hutchinson *et al.* 1992; Li *et al.* 1995; Pulford *et al.* 1995; Liu *et al.* 1993; Roberts *et al.* 1991; Spaete *et al.* 1993; Westra *et al.* 1997; Yaswen *et al.* 1993). The gL homologs appear to play an important role in transport and processing of their respective gH homologs. Complex formation between these two proteins is well established and it is generally assumed that this complex is essential for viral infectivity. Therefore the gH and gL homologs encoded by the newly identified gammaherpesvirus KSHV are most likely to exhibit analogous properties. Both nucleotide sequences ORF22/gH and ORF47/gL were PCR amplified and cloned into the mammalian expression vector pcDNA3.1 (Invitrogen). The amplification primers were designed to encode C terminal protein tags to facilitate protein detection; the c-myc epitope for ORF47/gL, and six histidine residues for ORF22/gH. The resulting clones pcDNA3.1-gL/pcDNA3.1-gH were transfected either singly or together into COS7 or HEK293 cells. The subsequent protein characterization was complicated as the amount of protein produced by these cell lines was very low, unless sodium butyrate was supplemented with the growth medium after transfection. In addition, several 293HEK stable cell lines, constitutively expressing ORF22/gH or ORF47/gL were established, which also synthesized low amounts of protein under 'non-induced' conditions. A basic

characterization of both viral glycoproteins was achieved, indicating several similar, but also some surprisingly different properties to other gH and gL homologs. It has to be emphasized that these expression systems may not reflect the situation in an *in vivo* herpesviral infection. The amounts of expressed protein in transient transfection systems is different and may not accurately reflect the amounts made during viral infection.

4.6.1 KSHV ORF22/gH and ORF47/gL expressed in mammalian cells

Both KSHV glycoproteins encoded by ORF22/gH and ORF47/gL could be successfully detected after chemical induction of 293HEK stable cell lines and transiently transfected Cos7 cells. The overall expression pattern within those two cell lines was similar, although glycosylation processes appear to be more efficient in 293HEK cells, in particular for ORF47/gL. Cos7 transiently transfected with ORF47/gL plasmids produced two proteins, 21 and 27kDa in size, which both reacted with the monoclonal antibody 9E10. Although no glycosylation studies have been performed it is likely that the 27kDa protein corresponds to the fully processed form, whereas the smaller protein may represent a precursor of ORF47/gL. Unlike HSV-1 gL which is secreted into the cell culture medium, both forms of KSHV gL were predominantly found in the soluble cell protein fraction. In 293 cells no reactive protein migrated at 21kDa, suggesting that this cell line performs more efficient post translational modifications. Cell-type dependent processing of gL has been reported before for the HSV-1 homolog, where gL precursor proteins could be detected in Cos and Vero cells, but L cells mainly

expressed the fully processed form (Dubin and Jiang, 1995). Furthermore, the results presented confirm observations with HIV-1 gp120, that 293 cells are able to perform efficient post-translational modifications (Fox *et al.* 1997).

In contrast, both cell lines seemed less able to perform secondary modifications of recombinant ORF22/gH. The synthesised product was identical in both cell lines, 293HEK and Cos7, when analysed by immunoblot. Cells produced an 80-85kDa protein that was found in the insoluble protein fraction. This protein is most likely to represent the precursor of gH, as the size corresponds to the predicted (unmodfied) molecular weight of ORF22/gH. Additional minor bands were observed with a molecular mass of approximately 110kDa, probably corresponding to processed form of gH. This reflects data reported for other herpesviral gH homologs where gH has been expressed as an insoluble protein which is incompletely processed when expressed alone (Westra *et al.* 1997; Pulford *et al.* 1995).

4.6.2 ORF47/gL and its chaperone function

As mentioned before herpesviral gH is dependent on the presence of gL for fully processing. When cells were co-transfected with gH and gL they synthesized a recombinant gH protein similar in size to the wild-type homolog, whereas singly transfected with gH the recombinant protein is smaller in size, apparently missing essential glycosylations (Foa Tomasi *et al.* 1991; Pulford *et al.* 1994; Gompels and Minson, 1989; Cranage *et al.* 1988; Heineman *et al.* 1988). Our co expression studies with ORF47/gL and ORF22/gH using our transfection system did not

result in either an increase in processed gH or in larger amounts of soluble gH gene product. This result suggests that for KSHV, gL appears to have less influence on gH than is seen in other herpesviruses.

Recently a third protein has been identified that associates with the gH-gL complex. So far this tripartite viral glycoprotein complex has only been described for HCMV and EBV. In EBV the third component has no obvious influence on gH-gL complex formation, whereas in HCMV the third protein, gO, was essential for the formation of mature gH-gL-gO complexes at the plasma membrane (Li *et al.* 1995, Huber and Compton, 1999). Neither gH nor gL were fully processed or could be detected at the surface membrane, unless gO was present. A similar situation may apply for KSHV, that is an additional as yet unknown protein may be an explanation for the lack of post-translational modifications of gH.

4.6.3 The Low expression level in both cell lines

Unexpectedly, the amounts of protein produced were low even when the transfection procedures were optimized. Studies using a control plasmid (pcDNA3.1-his-β-galactosidase) demonstrated an excellent transfection efficiency (50%) when transfected alone or in combination with ORF47/gL plasmids. Furthermore, expression levels showed no improvement when cells were cloned and stable cell lines established. Overall, we were unable to detect recombinant ORF22/gH and ORF47/gL by western blot without chemical induction, regardless which cell line was used. Only by collecting of a very large number of cells ($>10^7$) could recombinant protein be demonstrated, allowing estimates of the molecular

weight. Whether the recombinant protein itself or the promoter encoded by the vector was responsible for the low levels of protein remains to be determined formally. However, following transient transfections, a high number of detached cells and inhibition of cell growth could be seen, implying a possible toxic effect of these respective proteins. However, transient expression of herpesviral gH and gL homologs have been widely described with no major complications reported. As evident from IFA studies, which showed multinucleated cells following gL transfection, KSHV gL may exhibit properties that induce cell fusion and consequently cell death. Therefore surviving transfected cells may express only a limited amount of protein. Another possibility could be that the protein, or its c-myc epitope, is rapidly degraded during expression.

We were unable to investigate the intracellular distribution of ORF22/gH because of methodological problems, therefore it is difficult to explain the lack of expression in simple transient transfection experiments. We cannot exclude the possibility that gH may be retained in the endoplasmatic reticulum and be rapidly degraded. It could also be the case that gH is toxic and causes cell death. It has been reported that the establishment of stable cell lines expressing HSV-1 gH constitutively appears to be a rare event and in these cell lines the expression of gH seems low (Foa-Toamsi *et al.* 1991). Reduced gH expression has been demonstrated in HCMV strains that are resistant to neutralizing antibodies (Li *et al.* 1995), it has been suggested that this antibody-resistant phenotype can develop independently of genetic changes and maybe regulated by environmental factors (Li *et al.* 1995). Analyses of gH mRNA stability and the

promoter of HSV-1 gH have not provided any evidence for translational regulation. It is thought to be more likely that the expression of gH is dependent on post-translational events. This may explain why the expression of ORF22/gH in mammalian cells is complex and cells produce low amounts of protein. Further studies will be necessary, examining the intracellular distribution of ORF22/gH by indirect immunofluorescence, to address these problems of low expression as well as to directly investigate its influence on cell-to-cell fusion and gL surface expression.

5. Localization studies of recombinant KSHV ORF47/gL

5.1 Introduction

Herpesviral glycoprotein L has been described as a chaperone for gH processing and cell surface expression. When gL is expressed alone, in vitro studies showed that the protein is either secreted into the culture supernatant (HSV-1) or retained in the cytoplasm (EBV) (Pulford *et al.* 1995; Westra *et al.* 1997). This expression pattern is altered when co expressed with gH; gL and gH form a complex that can be identified at the cell surface. Recently, reports have identified a third component in HCMV and EBV that may be important during the transport of gH and gL. However, a possible homolog of this third protein has not yet been identified in KSHV. So far, the interaction of KSHV ORF22 and ORF47, the predicted gH and gL homologues, has not been studied. Using the established 293 stable cell lines and the transiently transfected Cos7 cells the intracellular distribution of ORF47 when transfected alone or with ORF22 was examined by confocal microscopy. Co transfection studies were mostly performed with Cos7 cells. However, the pattern observed was similar in both cell lines, (Cos7 and 293 cells) the KSHV gL homolog exhibited similar properties to other herpesviruses.

5.2 Initial IFA studies

Stable cell lines and transiently transfected Cos7 cells have been examined by IFA to determine expression efficiency (see chapter 4). This procedure was optimized and could be used for detail analyses of the intracellular distribution of ORF47/gL. However, the localization of gH when expressed alone, was problematic. The antibody penta-his was found to be unable to detect the histidine tag of a known control protein. Therefore, the expression of gH could only be demonstrated indirectly, assuming complex formation of gH and gL along with surface expression. Several experiments were initially performed testing various concentrations of primary (9E10, anti-c-myc) and secondary (FITC-conjugated anti-mouse) antibodies to optimize signal intensity (for optimal conditions see section 2.2.7.6). Experiments were repeated several times to assure that the results were reproducible and the protein could be detected in a similar pattern.

Other experiments were performed that addressed fixation and permeabilization of the cells. Different reagents were tested for their ability to fix and permeabilize cells. Methanol/acetone (1:1) produced the most consistent results, although 2% paraformadehyde in combination with acetone was similarly successful active. In contrast, 2% paraformaldehyde and Triton-X-100 resulted both in the loss of cells and in background fluorescence. As a consequence cells were routinely methanol/acetone fixed and stained as described before. Several hundred pictures were taken, a representative selection are presented in this chapter.

5.3 Intracellular distribution of ORF47, and its possible function

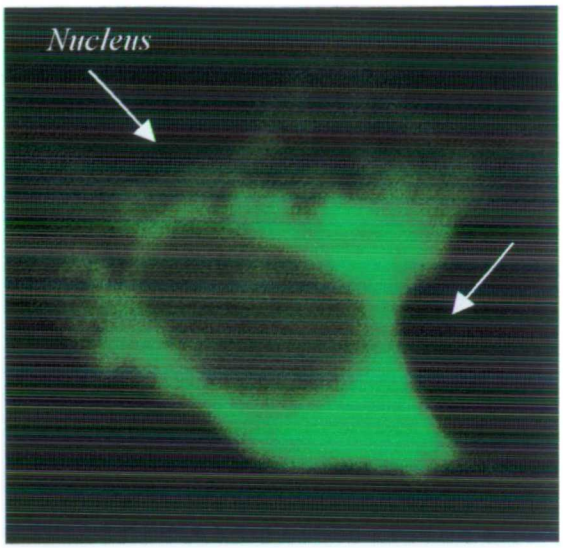
Cells transfected with gL plasmids were permeabilized and intense fluorescence observed in both transiently transfected Cos7 cells and in stable 293 cell lines. Overall, the fluorescence pattern in both cell lines was similar. Background fluorescence levels were low, as judged by the mock transfected Cos7 or 293 cells shown in figure 5.1C. When gL was transfected alone the c-myc-tagged gene product was detected in the cytoplasm, predominantly at the nuclear membrane, as shown in figure 5.1A. The fluorescent signal was localized and often shaped in a triangular pattern as indicated by the white arrow. This observation corresponds to other studies expressing EBV gL and VZV gL, where the protein is possibly retained in the ER (Pulford *et al.* 1995; Duus *et al.* 1996). Interestingly, in a minority of KSHV gL expressing cells the signal was distributed throughout the cell processes that attach to neighboring cells (see figure 5.1B). These data suggest that KSHV gL is not exclusively restricted to the peri-nuclear membrane.

Figure 5.1: Confocal microscopy analyses of c-myc tagged KSHV gL expression in mammalian cells. Transient transfected Cos7 or stable 293 cells were fixed in methanol/acetone and incubated with primary antibody 9E10 and a FITC-conjugated goat anti-mouse antibody.
(A) 'Triangular' staining pattern (see white arrow) in 293.5.10gL and Cos7 cells transiently transfected with pcDNA3.1-4.2gL
(B) Diffuse (cell process) cytoplasmic staining pattern in 293.5.10gL and Cos7 cells transiently transfected with pcDNA3.1-5.10gL
(C) Mock-transfected 293HEK cells

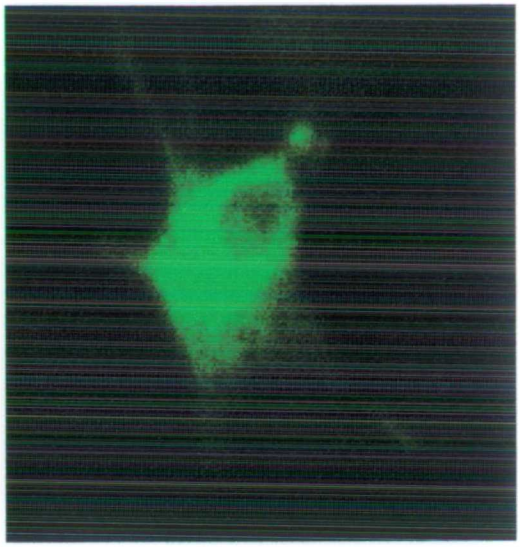
Figure 5.1:

(A)

293.510gL cell line

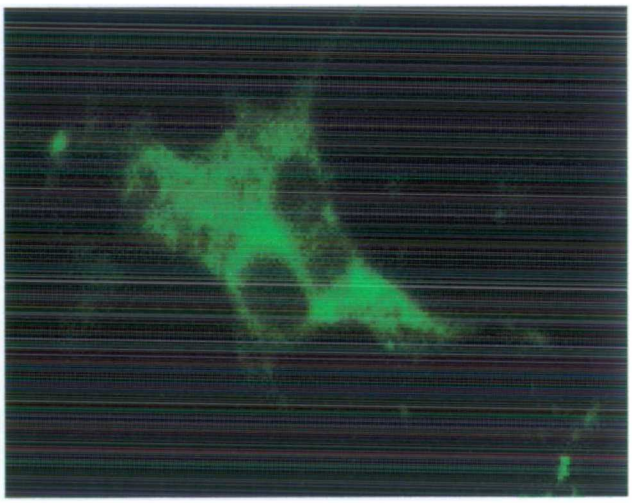


Cos7 cell line; 4.2gL

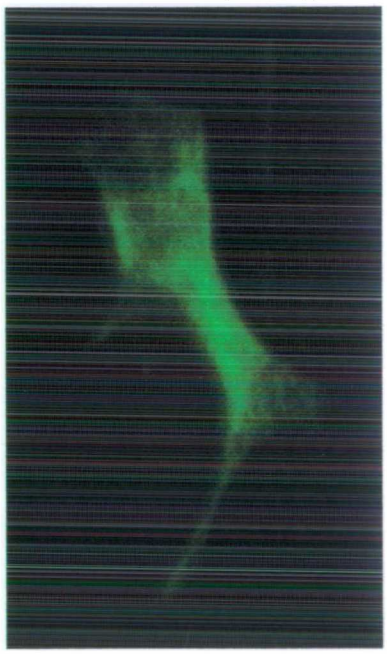


(B)

293.5.10gL cell line

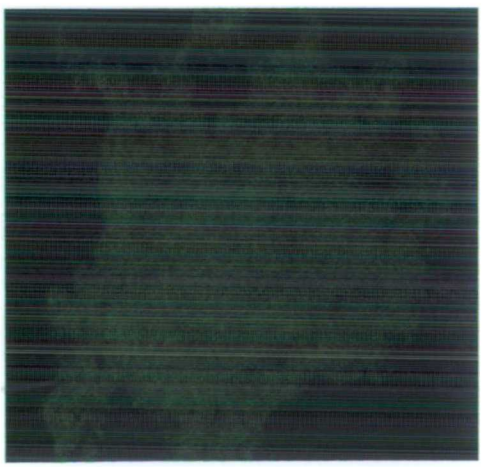


Cos7 cell line, 5.10gL



(C)

293HEK cell line



Moreover, the recombinant protein seems to traffic through the cytoplasm and may even be anchored to the plasma membrane, independently from gH. The possibility that KSHV gL can be found elsewhere than associated with the perinuclear membrane was further supported by the detection of multinucleated cells (figure 5.2). Although seen more frequently in 293HEK cells (panel A, B and C), Cos7 cells also exhibited this association of cells, some highly fluorescent (figure 5.2D). Some polykaryons showed a diffuse fluorescent pattern, in particular when a large number of cells were involved. In other multinucleated 293HEK cells, as shown in figure 5.3, a strong signal can be observed that is restricted to the surroundings of the nuclei, although faint signals can also be detected in areas towards the cell processes (see arrows). Polykaryons have been previously described in co expression experiments of herpesviral gH and gL. Those studies have shown that both proteins, glycoprotein H and glycoprotein L were required to mediate cell fusion. Cells transfected with either of the two proteins alone did not exhibit a fusogenic phenotype. It is therefore surprising that the KSHV gL appears to exhibit fusogenic properties. To directly induce cell to cell fusion gL has to be localized at the cell surface. Further experiments to examine cell surface expression of ORF47/gL could not confirm this assumption. Transient transfected Cos7 cells as well as 293 stable sell lines were analysed by several staining procedures, specifically targeting the cell membrane. However, neither staining live cells on ice nor staining non-permeabilized cells resulted in significant fluorescence intensity. In contrast, methanol/acetone fixed cells showed the usual staining pattern for gL expressing cells (data not shown).

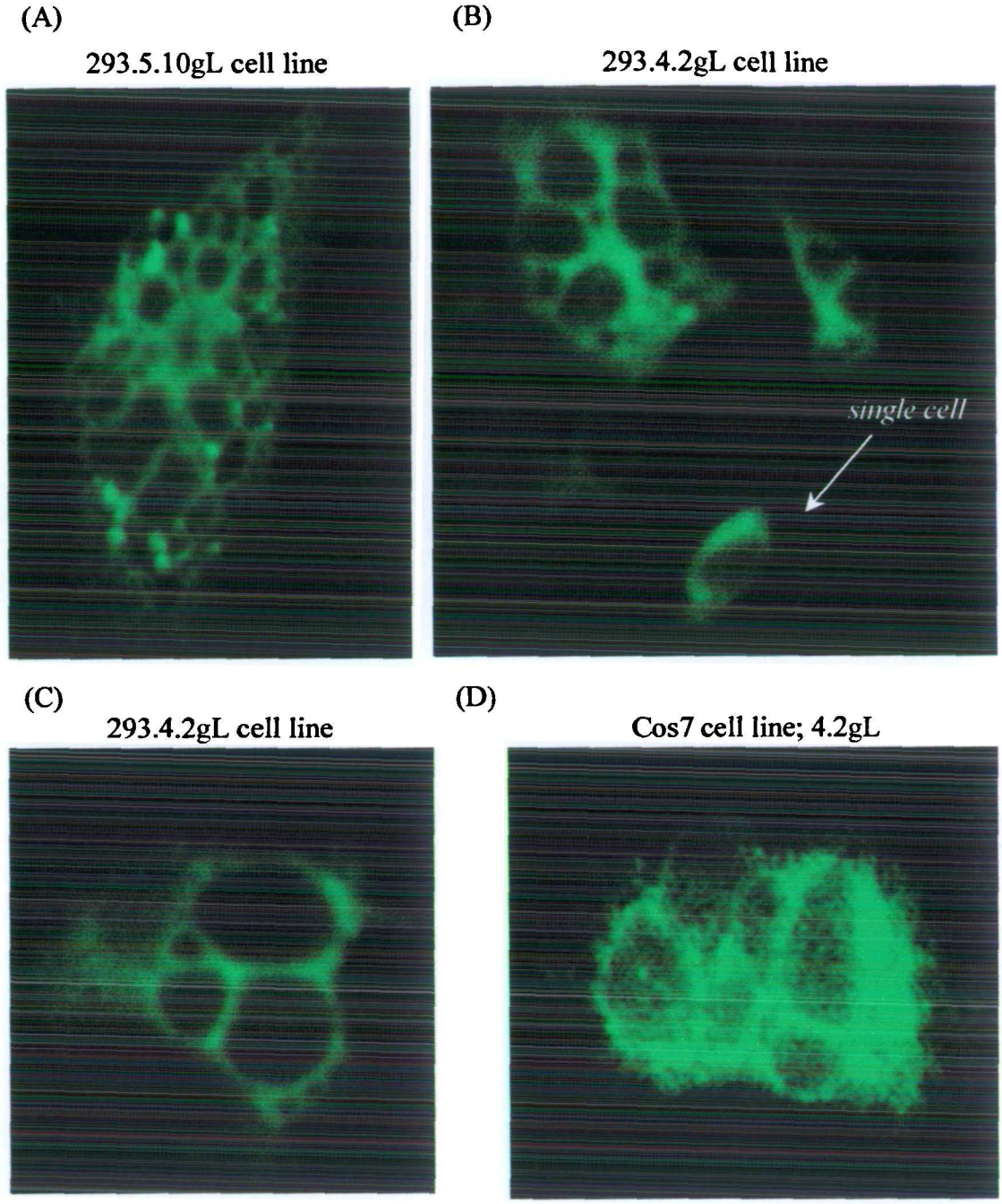


Figure 5.2: Confocal microscopy analyses of C-myc tagged KSHV gL expression in mammalian cells. Transient transfected Cos7 or stable 293 cells were fixed in methanol/acetone and incubated with primary antibody 9E10 and FITC-conjugated goat-mouse antibody.
(A) Multinucleated 293 cells formed by cells stably transfected with KSHV gL (5.10).
(B) Polykayron formation in 293.4.2gL, cells following butyrate induction. A single cell (white arrow) is shown in comparison to multinucleated cell.
(C) Polykayron detected in 293.4.2gL cells.
(D) Multinucleated cell formed by transient transfection of Cos7 cells with pcDNA3.1-4.2gL.

293.4.4gL cell line

293.5.10gL cell line

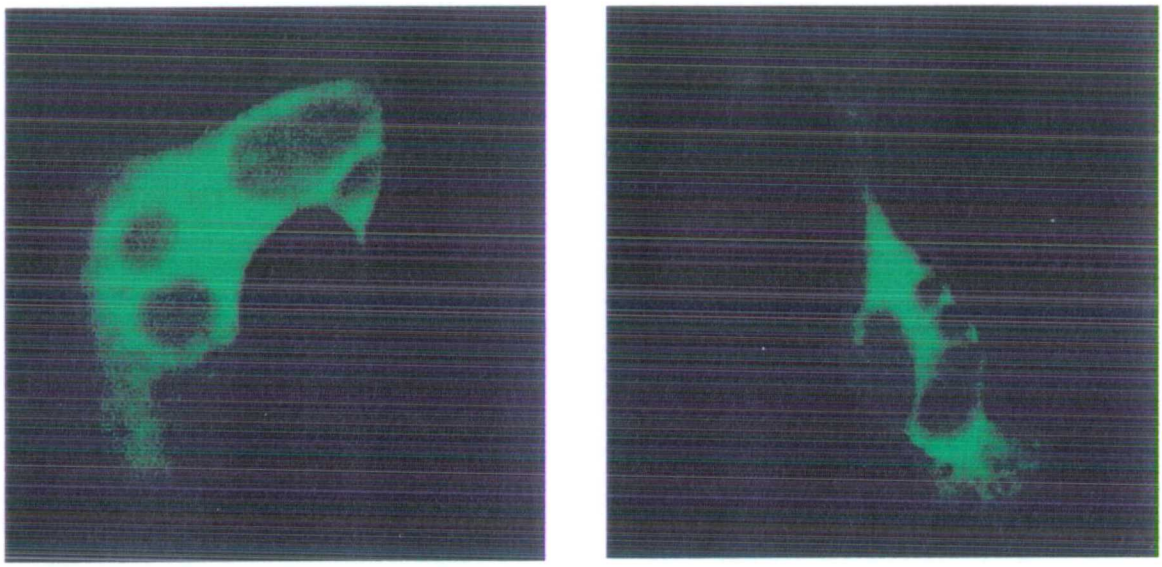


Figure 5.3: Multinucelated 293HEK stable cells (293.4.4gL and 293.5.10gL) exhibiting extensive perinuclear staining and limited trafficking into the processors (white arrows). Stable 293 cells were fixed in methanol/acetone and incubated with primary antibody 9E10 and a FITC-conjugated goat anti-mouse antibody.

However, the C terminus of the recombinant protein may project into the cytoplasm and hence the C terminal c-myc epitope was inaccessible to the primary antibody when stained under these modified conditions. To investigate gL surface expression an antibody specific for exposed N-terminal epitopes is essential.

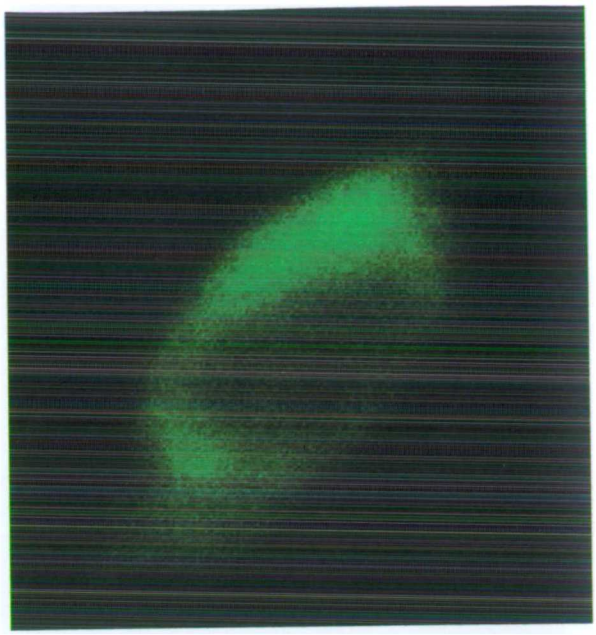
5.4 KSHV gL and Golgi organelles

Previous studies (chapter 4) suggested that recombinant KSHV gL is glycosylated, it therefore must traverse the Golgi compartments to be completely processed. To examine the localization of gL in more detail dual labeling experiments were performed using a fluorescent ceramide derivative that preferentially stains Golgi organelles (Bodiby FL-C_5 ceramide, Molecular Probes). Images are shown in figure 5.4. Cells were labeled as before and the dye added to the secondary FITC-conjugated antibody. Labeling of the Golgi was detected by examining cells with a filter that detects the red light emitted by the ceramide dye.

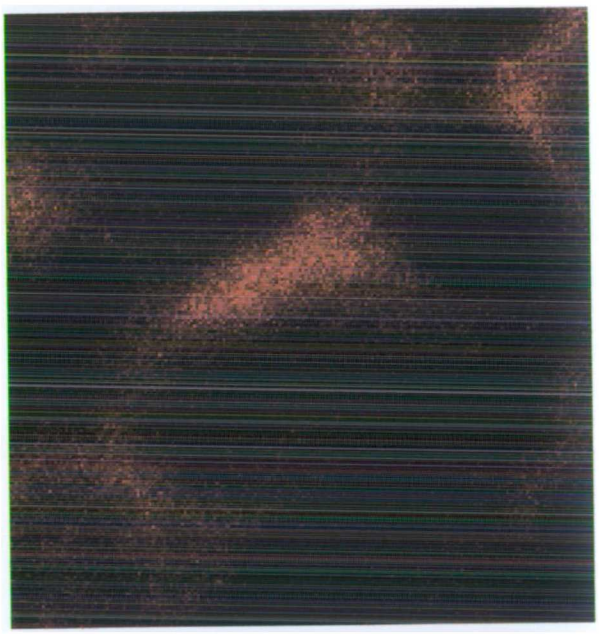
Figure 5.4: Co-localization of c-myc tagged KSHV gL with Bodiby FL-C_5 ceramide in the Golgi organelles of the cell line 293.5.10gL. Cells were grown on cover slips until 80% confluent. Following fixation cells were incubated with the Mab 9E10 and the FITC conjugated antibody added together with Bodiby FL-C_5 ceramide prior to examination by confocal microscopy. Single cells (A) and multinucleated cells (B) were examined.
(1) Green fluorescence emitted by the FITC-conjugated antibody, indicating gL expression
(2) Fluorescence emitted by the Bodiby FL-C_5 ceramide, represented by a red color and indicating Golgi organelles
(3) Co-localization of the red and green signals of panel 1 and 2, represented by a yellow color when electronically merged. (*): Due to technical problems the red and green color in this picture are inverted (see text for further details).

Figure 5.4A:

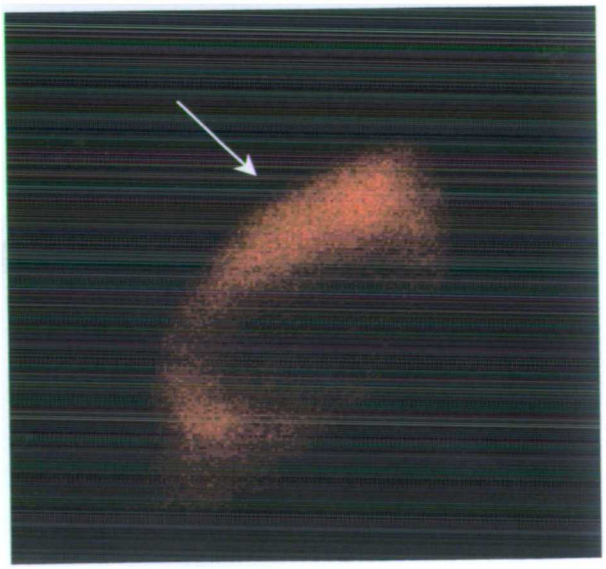
(1)



(2)



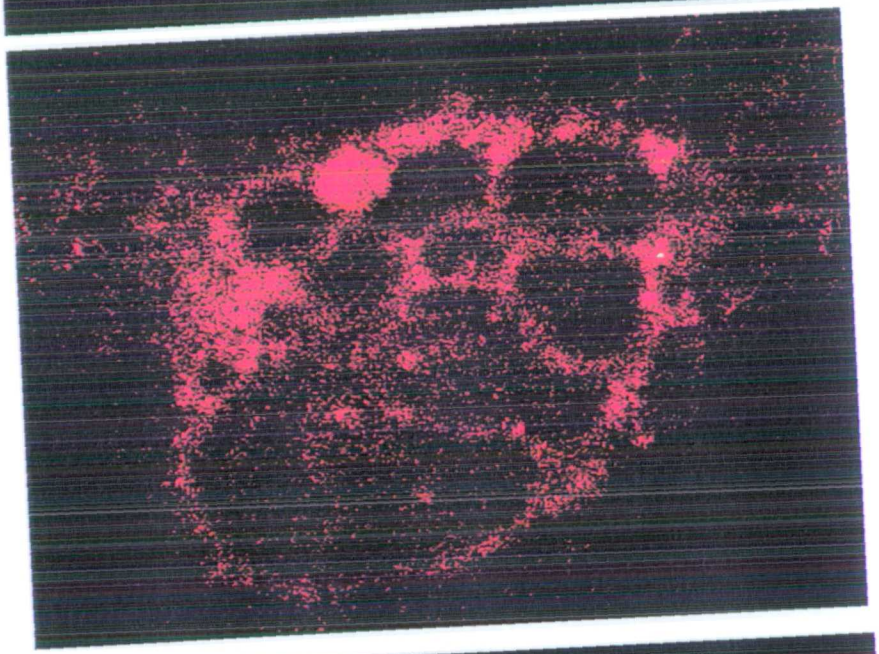
(3*)



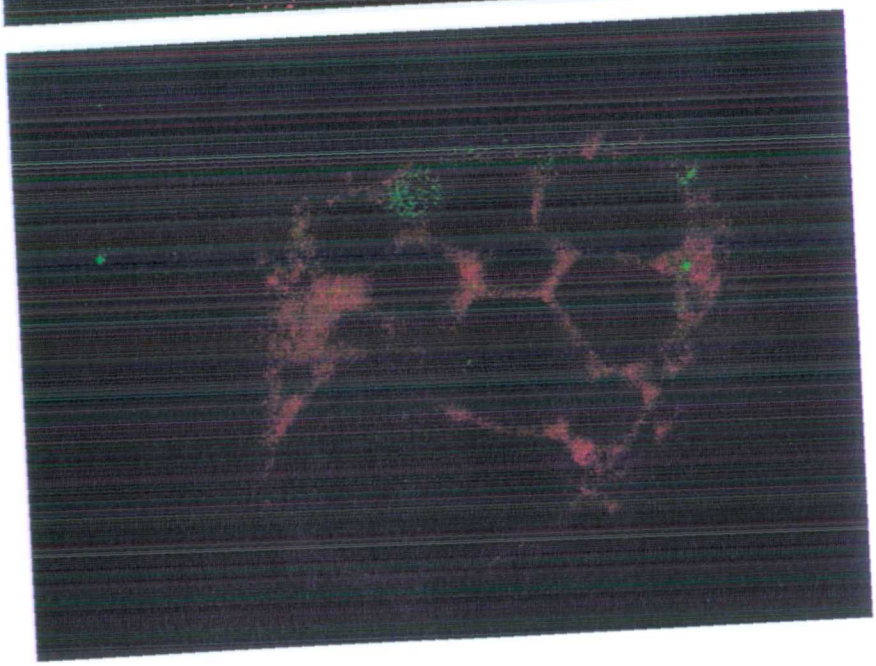
(B)

(1)

(2)



(3)



The same cells were immediately examined with a second filter that was used to detect light emitted by the FITC-conjugated secondary antibody. Two images were obtained and merged to form a two-color image. The orange/yellow colour in figure 5.4A3 and 5.4B3 represents areas where both signals co localize. Unfortunatly, we experienced some electronical problems while performing the color merge of the single cell, shown in panel A. During the color merge the two colors red and green were inverted, namely areas exhibiting green fluorescence (gL expression) are shown in red instead of green. However, despite this techinal problem a small area, colored in orange/yellow could be detected, indicating co localization of the two signals.

Several single cells as well as polykaryons were analysed and a general pattern could be deduced. Within a single cell (figure 5.4A) the yellow color in the cell shown in panel 3 is restricted to a small region of the entire area that is stained green by 9E10. Additional 'red' (supposed to be green) signal can be observed that did not colocalize with the portion stained by the dye (panel 1). This pattern suggests that gL is found in the Golgi compartment, but is possibly also retained in the ER, indicating the presence of pre-protein. In contrast, polykaryons, dually labeled with ceramide and 9E10, resulted in a different distribution of gL as shown in figure 5.4B. The yellow color in panel 3 can be more frequently detected than in single cells, indicating gL colocalization predominantly with Golgi organelles in polykaryons. This suggests that in these multinucleated cells gL is mostly present in its mature form.

5.5 Can ORF22/gH and ORF47/gL interact?

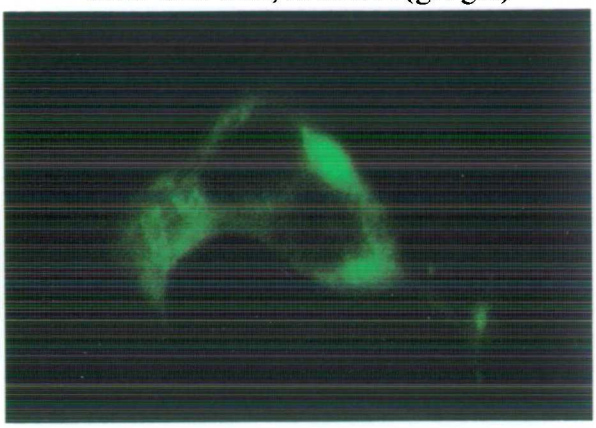
As described for other herpesviruses, gL is required for gH maturation and cell surface localization. The intracellular distribution of KSHV gL alone has been described above, whereas the recombinant expression of KSHV gH was limited to co-transfection experiments with both plasmids. However, efficient co-transfection of two plasmids is dependent on the ratio of the two constructs. It is hence rather unlikely that the two proteins will be expressed in the exact relative amounts required to only form complexes within the cell. Therefore several intermediate forms of expression patterns were expected, including diffuse cytoplasmic signals, cytoplasmic signals with surface expression, the triangular shape as seen before, etc. To determine just cell surface expression transfected cells were stained prior to fixation on ice without being permeabilized. This allows surface staining as endocytosis of the antibody is prevented, while cells are on ice. Also by staining live cells, rather than fixed/permeabilized ones, the recombinant protein remains in its native conformation. Possible changes in localization that may be induced by the fixative can be prevented. Co transfected cells stained on ice have been analysed by confocal microscopy, but were mostly negative, only a few cells could be detected that showed elevated fluorescence intensity, more or less restricted to the cell surface (data not shown). However, compared to cells stained following permeabilization, these fluorescent signals were insignificant. The molecular mechanism behind gH/gL complex formation has not been studied in detail for many herpesviruses A model of HSV-I gL complexed with gH has recently been described that would allow surface staining via the detection of a protein tag encoded at the C terminus of KSHV gL.

Truncated forms of gH and gL were co-expressed and the minimal region of both proteins required to interact defined. Together with data about the antigenically active sites a model was developed where the complex is anchored through the gH transmembrane domain and gL is localized extracellularly, with its N-terminus hidden within the confines of gH (Peng *et al.* 1998). However, whether this is the case for KSHV gH and gL is not known. The possibility that the C-terminal c-myc tag may be within the cell cannot be excluded (see above). Studies using antibodies specific for other regions of KSHV gL are necessary to address this question. Hence, cells were methanol/acetone fixed, stained as before at room temperature and examined by confocal microscopy for possible surface localization.

Results are shown in figures 5.5 and 5.6, illustrating different staining patterns. Strong fluorescent signals associated with the perinuclear membrane could be observed in most co transfected cells (figure 5.5A). In contrast, in a minority of cells the signal was diffusely distributed throughout the cytoplasm, as demonstrated by the Cos7 cells in panel B. Both patterns have been observed with cells expressing gL alone where, despite faint cytoplasmic staining, gL was strongly associated with the perinuclear membrane. However, some cells could be detected that did show an altered intracellular distribution when co transfected with gH and gL plasmids (figure 5.6). In particular 293HEK stable cells exhibited fluorescent signals associated with areas other than the peri-nuclear membrane. Unfortunately a green signal could not be localized to the cell surface in any cell analysed.

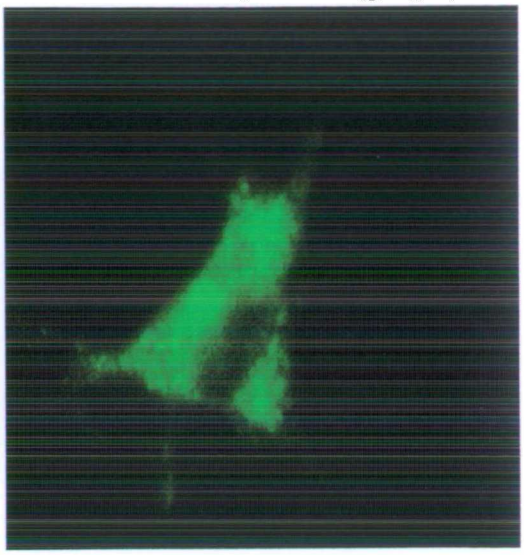
(A)

Cos7 cell line; 5.10/5.2 (gL/gH)

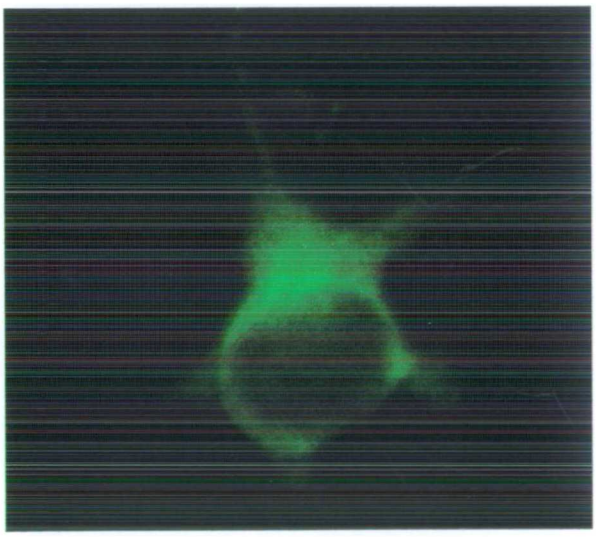


(B)

Cos7 cell line; 4.2/4.6 (gL/gH)



Cos7 cell line; 5.10/5.1 (gL/gH)



Cos7 cell line; 4.4/4.6 (gL/gH)

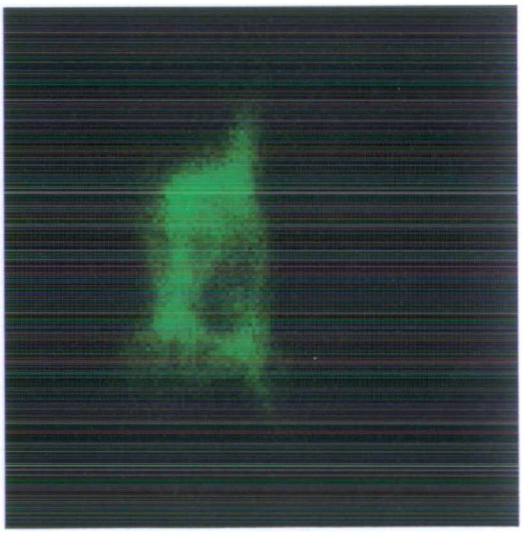
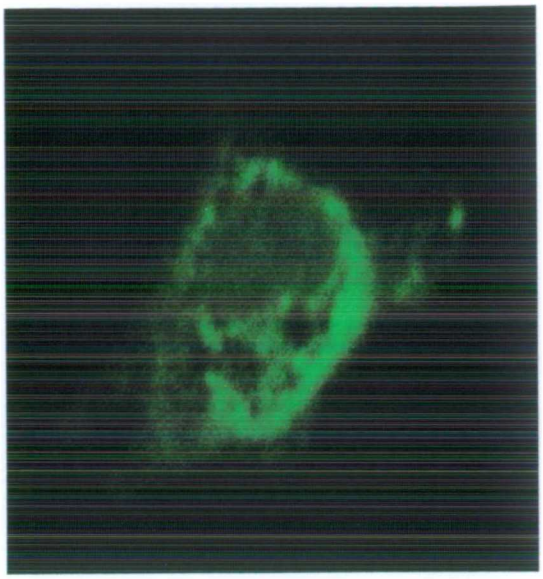


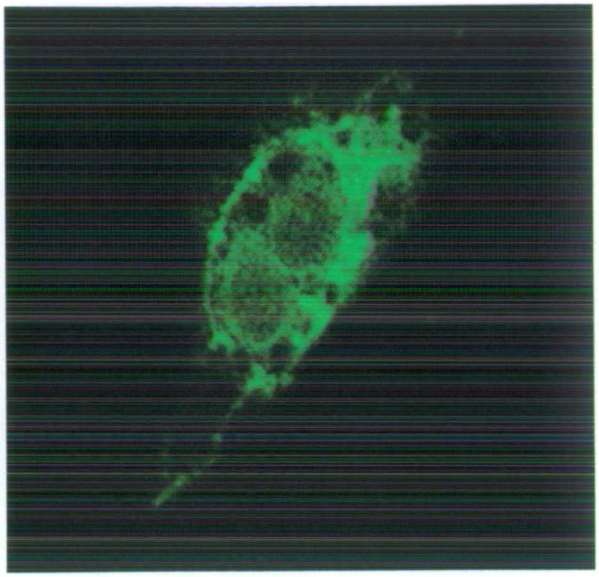
Figure 5.5: Confocal microscopy of Cos7 cells transiently co transfected with ORF22 and ORF47 plasmids exhibiting an 'intermediate' staining pattern. Cells were co-transfected on cover slips with a variety of expression constructs and analysed by IFA as described before using the Mab 9E10. Cells were then viewed by confocal microscopy. Transfected plasmids are indicated above each picture.
(A) Cells exhibiting peri-nuclear staining as well as cytoplasmic staining
(B) Cells exhibiting diffuse cytoplasmic staining

(A)

293HEK cell line; 5.10/5.2 (gL/gH)



293HEK cell line; 4.4/4.6 (gL/gH)



(B)

Cos7 cell line; 4.2/4.6 (gL/gH)

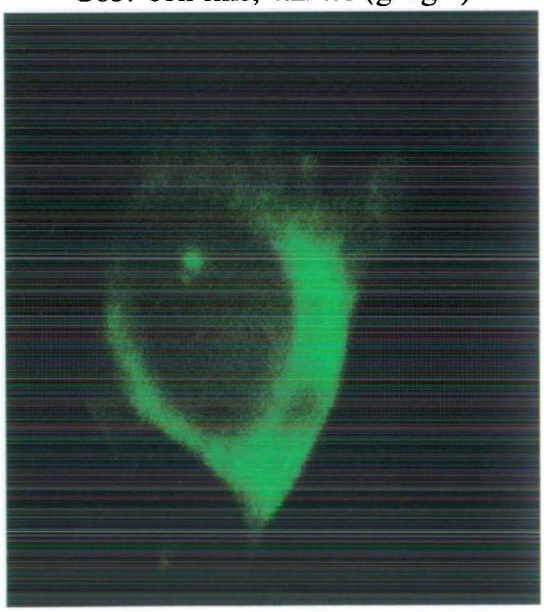


Figure 5.6: Indirect immunofluorescence staining of gH/gL co-transfected 293HEK and Cos7 cells. Cells were transiently transfected, methanol/acetone fixed and following 48 hours of incubation and analysed by IFA as described in the text. Cells were viewed by confocal microscopy. Transfected plasmids are indicated above each picture.
(A) 293HEK cells transiently co-transfected showing altered intracellular distribution.
(B) Cos7 cells transiently co-transfected, exhibiting diffuse staining pattern.

Whether the ORF22 gene product was directly responsible for this altered gL trafficking in co transfected cells is impossible to demonstrate without an anti-ORF22 antibody. We can only assume, as this pattern was absent from gL-expressing cells, that gH interacts with gL and causes this new distribution. A similar result was obtained with transiently co-transfected Cos7 cells. Most cells showed a diffuse cytoplasmic distribution when stained for gL expression (see figure 5.6B). Despite co transfection a difference in intracellular localization of gL could not be observed. To conclude, we were unable to demonstrate distinct surface expression of KSHV ORF47/gL when transfected along with ORF22/gH.

5.6 Dual labeling of co transfected cells

Similar to the analyses of gL transfected cells, co-transfected cells were examined for the co localization of gL and Golgi organelles. Cells were transiently transfected with gH and gL plasmids and stained as described before. Following labelling cells were analysed with different filters and images were merged to identify regions that were stained with both the antibody and the fluorescent dye. The number of cells successfully dually labeled and demonstrating altered intracellular distribution was limited. However, colocalization of the antibody 9E10 and Golgi organelles could not be detected, although cytoplasmic and mainly peri nuclear staining could be observed (data not shown). A series of experiments were performed but cells that showed a yellow color after the merge of two images could not be found, indicating that ORF47/gL did not colocalize with Golgi organelles when co-expressed with ORF22/gH.

5.7 Summary and discussion

Complex formation between herpesvirus glycoprotein H and glycoprotein L homologs is well established. It is generally assumed that the gH-gL complex plays a major role during penetration and cell-to-cell spread. So far, studies have shown that both proteins are necessary to mediate entry as well as fusion. Similarly, immunofluorescence studies have demonstrated that gL is required for processing and cell surface localization of gH. It has been reported that when expressed in insect cells from recombinant baculovirus, gH (HSV-1; HCMV) can be detected on the cell surface in the absence of gL (Ghiasi *et al.* 1991; Urban *et al.* 1996; Ghiasi *et al.* 1991). However, this has not been confirmed by others, who showed that gH only reaches the plasma membrane when gL is present (Westra *et al.* 1997). Unfortunately, the antibody we used to detect recombinant ORF22/gH reacted only weakly in immunofluorescence assays, so that corresponding experiments with KSHV ORF22/gH were not possible. Instead we focused on localization studies of KSHV ORF47, the predicted gL homolog, and monitored alterations of the gL distribution pattern when co-expressed with ORF22/gH. In the two different cell lines analysed the overall expression pattern was similar. Immunofluorescence studies confirmed western blot data showing that recombinant ORF47/gL is not secreted into the cell culture medium. ORF47/gL seems to be retained within the cytoplasm, in particular the ER, when expressed in mammalian cells. Confocal microscopy showed that KSHV gL can be associated with the nuclear membrane but also traffics through the cytoplasm and can be found in cell processes, when expressed individually. These observations corresponds to reports demonstrating that post translational processing and

intracellular transport of other herpesviral gL homologs in transfected cells do not require gH expression (Dubin and Jiang, 1995; Pulford *et al.* 1995). It is possible that the ORF47/gL product might be anchored to the cell surface via its amino terminal signal like sequence, although this has not been described for other herpesvirus gL products. Secretion of gL into the culture medium has so far only been reported for HSV-1, whereas for VZV and EBV recombinant gL is retained within the cell, most likely in the endoplasmatic reticulum (Pulford *et al.* 1995; Duus and Grose, 1996).

Interestingly, in our studies an association with the cell surface could not be demonstrated specifically, but the formation of polykaryons was observed, suggesting that some gL may be localized to the cell surface and induce cell to cell fusion. This observation is rather surprising as in other herpesviruses gH homologs have usually been described as the key factor in herpesviral induced cell to cell fusion. Neutralizing antibodies specific for gH inhibited HCMV induced cell fusion (Milne *et al.* 1998). Others have found residues within the C terminal tail of HSV-1 gH that are essential for fusion events (Wilson *et al.* 1994). However, limited data is available that addresses the function of gL homologs in cell to cell fusion. Studies using a herpesvirus mutant, where gL is deleted, showed that in HSV-1 gL is similarly dependent on gH for viral infectivity (Roop *et al.* 1993). Furthermore, antibodies against HSV-1 gL have been shown to effectively inhibit cell fusion, indicating a role for gL in this process (Novotny *et al.* 1996). There has been evidence that co expression of EBV gL and gH may not be required for cell surface transport of gL, but a fusogenic phenotype depends on

the expression of both (Li *et al.* 1995). It is hence possible that the gL homolog encoded by KSHV may exhibit fusogenic properties.

Since we were able to detect the KSHV gL homolog by confocal microscopy we decided to examine whether gL co localizes with certain cellular organelles, particularly the Golgi. Secretory and membrane proteins are synthesized by ribosomes bound to the ER. From there glycoproteins are transferred to the Golgi organelles, where the trimming and terminal glycosylations of the oligosaccharides units takes place (Stryer, 1995). Therefore, fully processed gL will traverse through the Golgi during maturation. Confocal microscopy examining the association of gL and Golgi organelles showed differences between single cells and polykaryons. In single cells that express gL, only a small area can be detected that co localizes with the Golgi, whereas the majority of the protein is associated with the nuclear membrane, most likely with the ER, as indicated by the shape of the fluorescent signal (Lippincott-Schwartz *et al.* 1990). In contrast, polykaryons show a reverse pattern, with the fluorescent signal being mainly found in the Golgi. These data leads us to speculate that a proportion of cells express gL as a possible inactive precursor protein and therefore remain as single cells. Other cells, however, may produce the fully glycosylated form of gL which travels through the Golgi and is able to induce the formation of multinucleated cells and consequently lead to cell death. This hypothesis would help in explaining the low amount of protein produced by both mammalian cell lines, described in chapter 4. Cellular over-expression of mature ORF47/gL gene product would be disadvantageous to the cell because the gL protein, apparently, encodes fusogenic

domains and causes cell death. Therefore, surviving cells may have down regulated this expression, with only a minority of cells expressing the protein. Overall the findings that gL may be involved in fusion are interesting, as gL has so far been identified as a chaperone-like protein only, essential for proper processing and targeting of fusogenic gH. In our studies KSHV gL seems to have a more active function and appears to directly contribute to cell-cell fusion.

During our experiments localization studies of ORF22/gH were impossible as the antibody penta-his did not recognize the C terminal histidine tag in indirect immunofluorescence studies. Therefore intracellular distribution of gH could not be examined, thus its possible cell surface expression or retention in the cytoplasm when expressed alone remain to be determined.

5.7.1 Do the gene products of KSHV ORF22/gH and ORF47/gL form a complex?

Co-expression studies were predominantly performed in Cos7 cells transiently transfected with ORF47/gL and ORF22/gH plasmids. Different ratios of the plasmids were assessed and showed a variety of expression patterns. We were unable to obtain cell surface expression and the results shown are only based on the detection of gL. However, the intracellular distribution of gL was altered in some cells when co expressed with gH. Fluorescent signals were localized to other areas than the nuclear membrane. Most cells showed the fluorescent pattern seen in cells transfected with gL alone. Therefore the pattern seen in some co-transfected cells could be due to gL being expressed alone. We were unable to

detect gH by confocal microscopy for the reasons discussed and therefore cannot exclude that gH may not be involved directly in gL trafficking. Western blot analyses showed no significant difference in molecular weight for either proteins when co expressed. There may be some increase in fully processed ORF47/gL when co expressed with ORF22/gH, but this result could not be consistently quantified.

As mentioned in chapter 4 the fact that a third protein associates with the γ-herpesvirus EBV gH/gL complex may imply a similar scenario for KSHV. That is cell surface expression of KSHV gH/gL may be influenced by a viral protein yet to be identified.

To conclude, recombinant KSHV ORF47/gL gene product, produced in mammalian cells can be found within the cytoplasm and may be involved in cell to cell fusion. Our studies were limited to the detection of C-terminal protein tags engineered during PCR amplification, confirmation with monoclonal antibodies specific for the individual proteins would be desirable. Also, the lack of direct gH detection in immunofluorescence studies made it difficult to analyse the interaction between gH and gL in more detail.

6. Propagation of KSHV in the hepatoma cell line HepG2

6.1 Introduction

The molecular pathogenesis of KSHV has remained elusive, as *de novo* infections are rare. Several B cell lines derived from PEL patients have been established that harbor the virus. The majority of these cells maintain a latent KSHV infection and, following treatment with phorbol ester or sodium butyrate, support lytic replication. However, all cells in these culture systems are already infected, making it impossible to study viral infectivity. Attempts to transmit infection to other cell lines by inoculation with B cell derived material have been made. Many cell lines of fibroblastic, epithelial, endothelial or lymphoid origin were susceptible for KSHV, but infection was both inefficient and abortive, the KSHV genome being lost upon passage. Recently, some endothelial based cell culture systems have also been described that were stably infected with KSHV and developed a spindle cell shape resembling KS spindle cells in vitro. For a detailed background of the propagation of KSHV in vitro see chapter 1. To test the fusogenic properties of the gH/gL complex of KSHV we were interested in finding a possible target cell of KSHV. From our experience of maintaining BCP-1 cells in culture we identified the human hepatoma cell line HepG2 as a useful feeder layer that increased viability of BCP-1 cells. Moreover, it was noted that in some BCP-1 feeder layers HepG2 cells developed aberrant morphology. Hence, the susceptibility of HepG2 cells for KSHV infection was tested. Cells were inoculated with KSHV derived from a singly infected B cell line (BCP-1) and with virus obtained from SY cells which are infected with both

KSHV and EBV. At different time points during incubation HepG2 cells and supernatant fluid were analysed by PCR for the presence of KSHV specific DNA. Surprisingly, we were only able to detect viral DNA over an extended period of time when inoculating with virus derived from SY cells, suggesting a possible influence of EBV. However, after only a few passages the viral genome was not maintained, indicating an abortive infection.

Infection experiments were performed several times and the results were consistent. These sets of data are discussed below.

6.2 The KSHV donor cell lines: BCP-1 and SY

The KSHV positive cell line, BCP-1, has been established from the peripheral blood of an HIV sero-negative patient with PEL (Boshoff *et al.* 1998). BCP-1 cells injected intraperitoneally into mice were demonstrated to induce the development of ascites and diffuse infiltration of organs, without obvious solid tumor formation, this pathology resembles the primary effusion lymphomas seen in humans. BCP-1 cells are singly infected with KSHV and contain on average 30-50 KSHV genomes per cell (Howard M, PhD thesis, 1999). The second cell line, SY, is derived from the ascitic fluid of an HIV positive patient with PEL. Following nested quantitative PCR, KSHV and EBV sequences have been detected in a 150 to 1 ratio (personal communication, U. Ayliffe). The cell line has been established in our laboratory; but further characterizations have not been performed. Both cell lines, BCP-1 and SY, showed only 50% cell viability, judged by trypan blue exclusion, when propagated in complete RPMI culture medium. An

improvement in cell viability was observed when these cells were maintained in association with a feeder-monolayer of human embryonic lung cells (HEL) (M Howard, PhD thesis 1999). Therefore, cell cultures of B cells derived from individuals with PEL were routinely co-cultured with a monolayer of HEL cells. Interestingly, apoptotic BCP-1 or SY cells remained attached to the feeder layer, whereas healthy cells were detected in suspension (M Howard, PhD thesis 1999).

Both cell lines were positive by nested PCR for the presence of KSHV. The SY cells were also positive for EBV (data not shown).

6.3 The recipient cell line: HepG2

The human hepatoma cell line, HepG2, exhibits several features of normal hepatocytes. This cell line is well differentiated and retains the ability to synthesize hepatic proteins (Bouma *et al.* 1989) and has been the target for several viral studies, including Hepatitis B virus receptor studies together with HCV and HHV6/HHV7 infection studies (Mabit *et al.* 1996; Seipp *et al.* 1997; Garson *et al.* 1999; Cermelli *et al.* 1996). HepG2 cells appear to be permissive for infection by other herpesviruses, such as HHV6/HHV7 and their replication can be induced by treatment with phorbol esters (Cermelli *et al.* 1996). EBV, the closest human homolog to KSHV, has been associated with liver disease during infectious mononucleosis and post-transplantation lymphoproliferative disorders (Markin, 1994). For these reasons HepG2 cells may be a possible target for KSHV infection. Furthermore, we have shown that HepG2 cells can function as a feeder-

monolayer for the maintenance of BCP-1 cells, similar to HEL cells (personal communication, U. Ayliffe).

Prior to infection studies, nested PCR of HepG2 cells was performed to assess the presence of other herpesviruses. Cells were negative for HSV-1, HSV-2, HCMV, VZV, HHV-6, KSHV and EBV (data not shown).

6.4 Transient Infection studies

To determine HepG2 susceptibility to KSHV, transient infection studies were initially performed. Since BCP-1 cells are singly infected with KSHV, this cell line was used as the source of viral particles. Cell-free supernatant was prepared from fresh BCP-1 cell cultures by centrifugation of the supernatant for 30 min at 5000rpm. This supernatant was added in serial dilutions to HepG2 cells grown in six well dishes, aliquots (200µl) of the inoculum in each well collected and the viral genome copy numbers assessed by limiting dilution PCR (day 0). Copy numbers ranged from 10^7 to $6x10^7$ per well. As a negative control cells were mock-infected. All cell monolayers were exposed to the inoculum for a total of 7 days.

Aliquots (200µl) of supernatant fluid were collected at 1 day post-infection (PI), 4 days PI and 7 days PI. On day seven supernatant was removed, cells washed three times in PBS and the final wash as well as 10 000 cells harvested (7 days PI). Total genomic DNA was extracted from all samples and KSHV infection assayed by nested qualitative and quantitative PCR.

6.4.1 The nested qualitative PCR

The nested PCR for the detection of KSHV specific DNA was established previously as part of M. Howard's PhD theses (1999). Oligonucleotide primers were designed to detect the viral DNA fragment, KS330Bam, described in the original identification of KSHV (Chang *et al.* 1994). The first round primers specifically amplify this fragment, which is part of the KSHV ORF26 (minor capsid gene). Sequence analyses of KSHV strains showed that genetic variation of ORF26 is low, allowing the design of a nested PCR (Zong *et al.* 1997; Zong *et al.* 1999). Thus, a primer set sited internally to the already published primers was designed enabling the amplification of a 172bp DNA fragment (M. Howard, PhD thesis 1999). PCR conditions were optimized and the sensitivity of the amplification assay assessed using viral DNA purified from a KSHV-containing B cell line. Consistent with other researchers, who also designed a nested PCR specific for ORF26, we determined the sensitivity as 10 copies of the viral genome (Boshoff *et al.* 1995; M Howard, PhD thesis, 1999). However, on occasions it was possible to obtain limited sensitivity, detecting one copy of the viral genome (see chapter 7, figure 7.3). Oligonucleotide primers are listed in chapter 2.2, along with a detailed description of the amplification protocol. A more detailed description of the ORF26 sequence variation among different KSHV isolates is discussed in chapter 7, as part of the establishment of a KSHV quantitative assay.

6.4.2 HepG2 cells are permissive for KSHV

Gel electrophoresis of transient infection samples, shown in figure 6.1A, demonstrated that the PCR yielded consistently strong signals for each sample, including supernatant, wash and cell lysate. The quantity of virus added to the cell monolayer was less significant, as any dilution produced similar results. In addition to the supernatant being positive for KSHV throughout the experiments, HepG2 cells, harvested 7 days post-infection (PI) also contained viral DNA (indicated by C for cells in panel A), although with a reduced signal intensity. This result may not reflect internalized DNA, as the third wash (W) was also positive by PCR. It is hence possible that KSHV may associate with the cell surface and can therefore be detected by PCR when analysing the HepG2 cell lysate. Subsequent quantification of the viral copies within the 'washing' sample and the cell lysate using a quantification assay (ELONA) revealed that a difference in the copy number between third wash and cell lysate did exist. These data are graphically illustrated in figure 6.2, black bars indicate the number of copies identified within the cell lysate, the third wash samples are represented by white bars. Wash samples only contained a few viral copies (<50 copies/μl), whereas cell lysates harbored many more viral sequences (up to 600 copies/μl). The quantification assay will be described in detail in chapter 7. Briefly, a single round PCR was performed and the amplification products were compared to standards of known copy number by means of a chemiluminescent reaction. Following the fitting of a regression line to these data, copy numbers were estimated by interpolation.

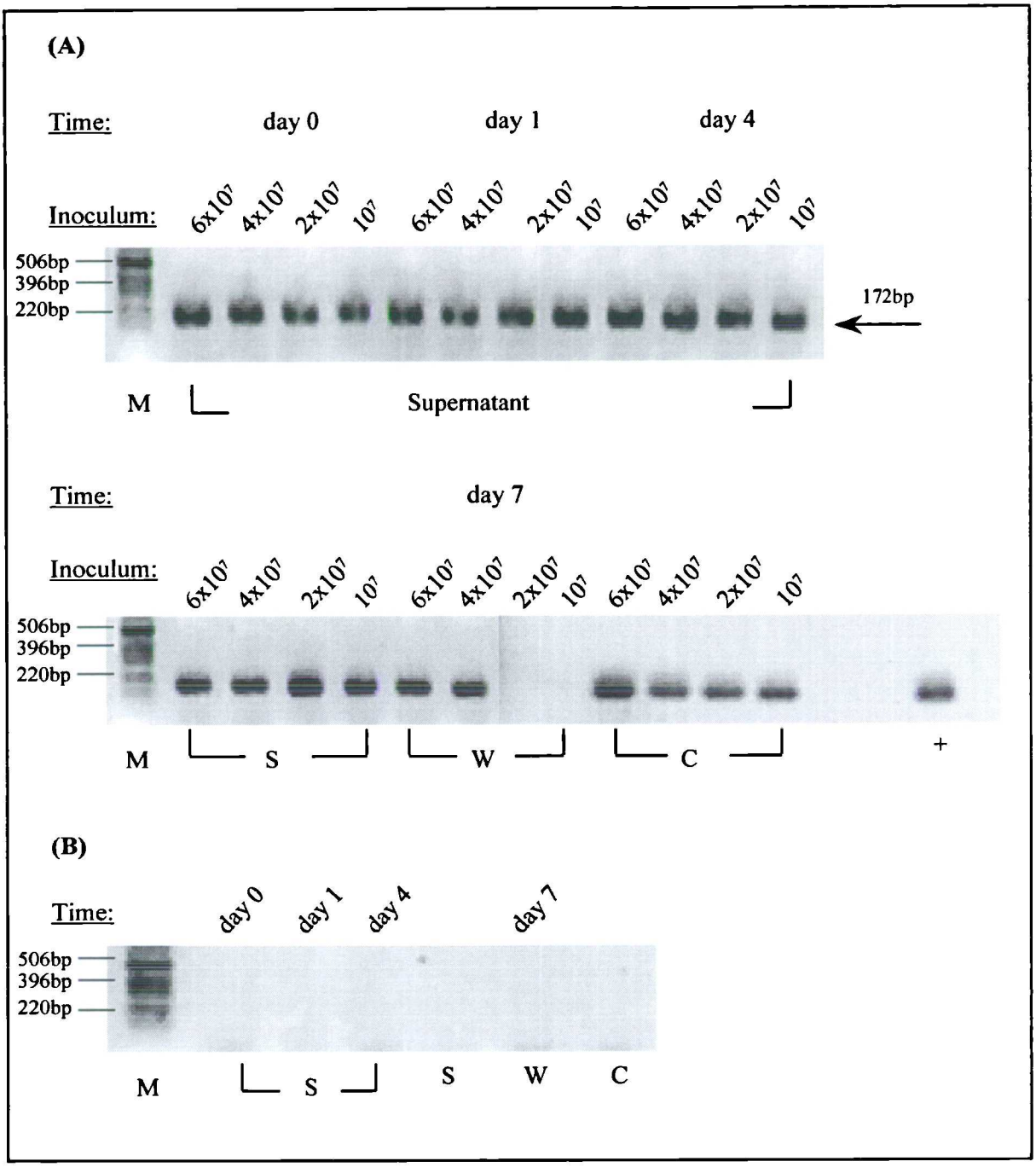


Figure 6.1: Detection of KSHV DNA sequences in HepG2 cells following 7 days exposure to BCP-1 cell culture supernatant. HepG2 cells were seeded in 6 well dishes and inoculated with serial dilutions of supernatant (indicated above each lane). Samples were harvested at the days indicated and analysed by nested PCR as detailed in the text. In panel A an arrow indicates the KSHV specific 172bp DNA fragment, (+) indicates positive control sample (BCP-1 supernatant); (M) denotes molecular size markers.
(A) Samples obtained from inoculated cultures.
(B) Samples obtained from mock-infected HepG2 cells.
(S) supernatant fluid; (W) third PBS wash; (C) cell lysate.

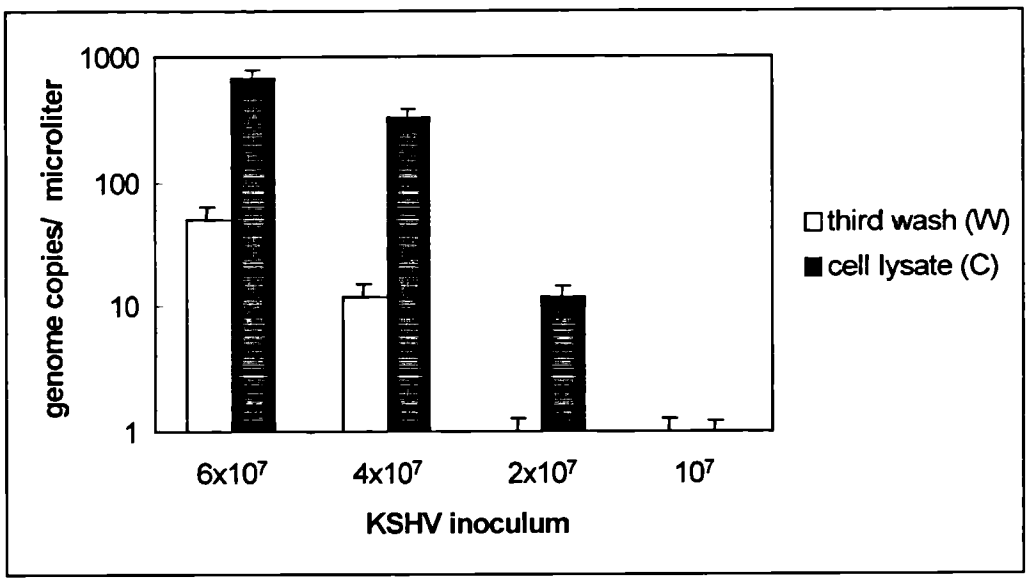


Figure 6.2: Quantification of viral DNA present in third wash and cell lysate samples that have been inoculated with KSHV. Samples were collected 7 days post-inoculation and subjected to a single round of PCR, followed by a 96 well based quantification assay (ELONA, see chapter 7). Data obtained was analysed using the computer software Genesis Version 2.20. White bars indicate third wash samples; black bars illustrate the amount of viral amplicons found in cell lysate.

As a negative control, non-infected HepG2 cell cultures were treated similarly and showed no viral DNA signal following qualitative (figure 6.1B). PCR. All experiments were repeated and performed in duplicate.

In summary, this result suggests that HepG2 cells can be transiently infected with KSHV derived from BCP-1 cells. KSHV may cell-associate but only a minority of virus appears to be able to infect HepG2 cells. To establish whether KSHV can be serially transmitted in HepG2 cells, long term propagation studies were performed and the presence of viral DNA monitored over time by nested PCR.

6.5 Serial Passage of KSHV in HepG2 cells

Transient infection of KSHV has been demonstrated for a variety of different cell lines, we were interested whether HepG2 cells could maintain the viral genome over an extended time period. Two separate experiments were performed using virus derived from two different B cell lines.

A) KSHV derived from the BCP-1 cell line, infected with only KSHV.

B) KSHV derived from the SY cell line, dually infected with both KSHV and EBV.

This second cell line was selected in response to recent studies reporting that the presence of EBV may influence KSHV infection of B cells (Kliche *et al.* 1998).

The experiments were performed under identical conditions and repeated several times. Initial time-course experiments were performed, passaging cells after 3 days and 7 days exposure to virus. PCR analyses showed that an extended period of infection (up to 7 days) was necessary to obtain a positive PCR signal within HepG2

cells. Three day studies demonstrated that viral DNA could be infrequently detected in some samples prior to the first passage, including supernatant (S), third wash (W) and HepG2 cells (C) harvested three days PI (see figure 6.3). The amount of virus added initially appeared to play a minor role, as HepG2 cells exposed to a high viral titer (10^8) could not be infected repeatedly. However, these PCR signals were lost in every sample tested after the cells had been split, both by analysing 1µl or 10µl (data not shown) in a nested PCR. Since viral DNA was absent from wash samples and lost after passage, HepG2 cells appear to be susceptible to low level infections. However, it cannot be excluded that the initial positive signal within the HepG2 cells may have been due to cell-surface associated KSHV DNA and these residual KSHV particles may have contaminated the cell lysate samples.

In contrast, seven day experiments were more successful and are discussed below. As a consequence of these observations, in all studies the inoculum was removed from each long term infection study after 7 days of incubation. The infection protocol was as follows: an equal number of HepG2 cells were transferred to four tissue culture flasks (T25) and grown to 50-60% confluence. BCP-1 or SY supernatant was harvested as described above and HepG2 cells inoculated with different dilutions of the cell-free supernatant. Flask 1 was overlaid with a BCP-1 or SY supernatant containing 10^8 copies; flask 2 was exposed to $5x10^7$ copies and flask 3 to $2.5x10^7$ viral copies. As a negative control, a 4th flask of HepG2 cells was incubated with culture medium alone and treated similarly to the infected flasks. Aliquots of the supernatant were taken immediately after inoculation, 3 days and 7 days PI. Seven days post-infection the inoculum was removed and cells split in a 1 in 2 ratio (see arrow

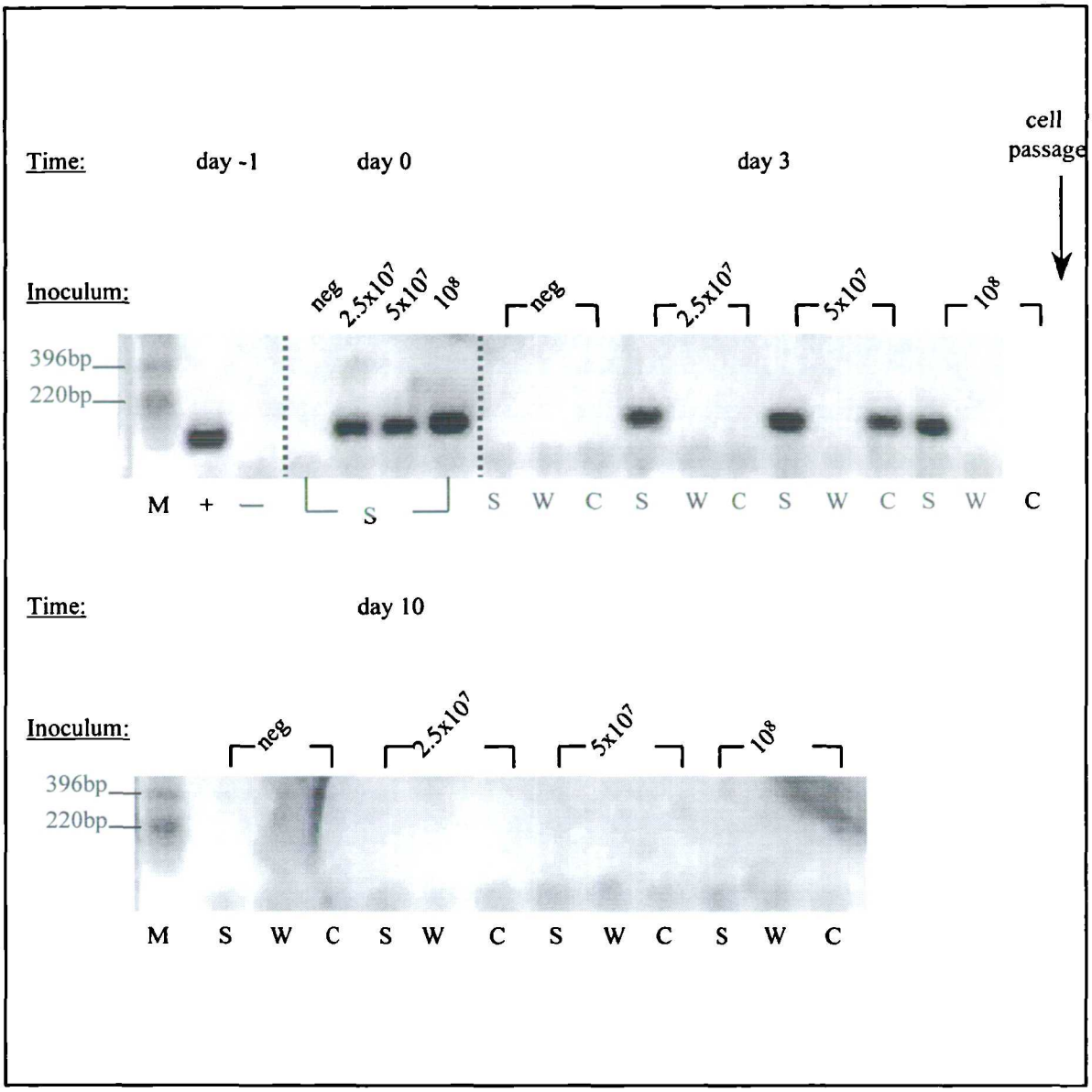


Figure 6.3: Serial passages of HepG2 cells inoculated for 3 days with supernatant fluid obtained from BCP-1 cell cultures. HepG2 cells were grown in tissue culture flasks and inoculated with different amounts of KSHV particles, as stated above each lane. Samples were collected over a period of 5 to 7 weeks and total DNA extracted and tested by nested PCR. Days post-infection are noted. Time of cell culture passages are indicated by arrows. S: cell culture supernatant; W: third wash sample; C: Cell lysate sample; (+) positive control (BCP-1 supernatant); (-) negative control (non-infected HepG2 cell lysate).

indicating cell culture passages), transferred to a new culture flask and incubation continued until cells were confluent (approximately one week). Prior to each passage samples were taken, including aliquots of supernatant, final wash and cells. Total DNA was extracted from each sample (200µl, or 10 000 cells resuspended in 200µl of PBS) and one or ten microliters analysed by nested PCR. Amplicons were analysed on a 1% agarose gel for the presence of the 172bp fragment (figure 6.5 and 6.6). Negative and positive controls were included in all PCR reactions. As a positive control a PCR mix contained 1µl of BCP-1 supernatant was used.

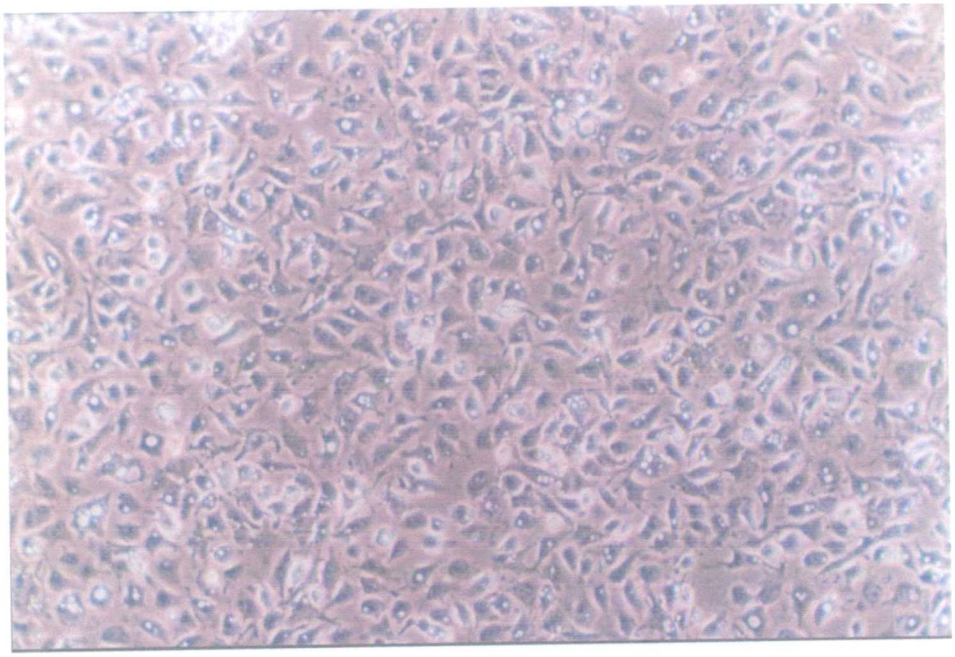
6.5.1 KSHV infection using supernatant derived from BCP-1 cells

6.5.1.1 Phenotypic changes of inoculated HepG2 cells

Supernatant, third wash and cell lysate samples were collected during the experiment and subsequently examined by PCR (see below). Interestingly, an increase in cytotoxicity could be observed in cells incubated with B cell derived supernatant for 7 days. During culture HepG2 cells started to round up, detach from the plastic and clump together, suggesting apoptotic cell death. The majority of these cells remained attached to the cell monolayer, resembling the apoptotic BCP-1 cells that attach to HEL monolayers. In figure 6.4 mock-infected HepG2 (A) cells together with KSHV infected cells (B) are shown. A white arrow indicates aggregated HepG2 cells. Some dead cells could be detected within the mock-infected control flask as well, but compared to KSHV infected cells, no aggregation was observed.

On day 7 the inoculum was removed, cells washed and harvested as described before. Samples were stored for PCR testing and cells seeded into new flasks. Following the

(A)



(B)

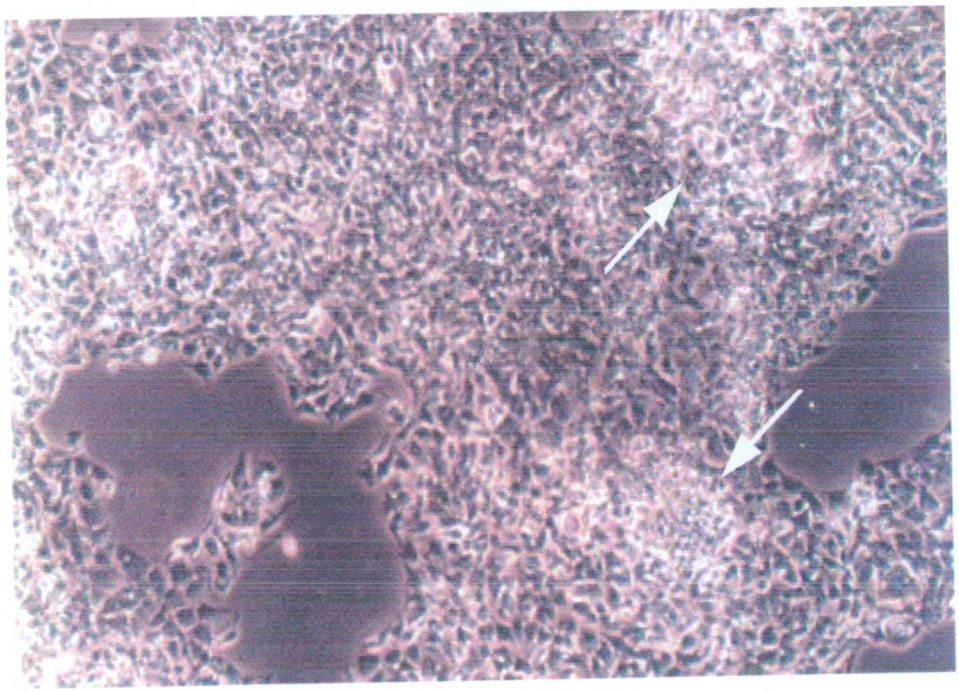


Figure 6.4: Cytotoxic effects on HepG2 cells that have been infected by KSHV *in vitro*. Panel A shows uninfected HepG2 cells after incubation for the numbers of days shown. Panel B shows the appearance of KSHV inoculated HepG2 cells after being passaged once. The blue arrow highlights detached and aggregated cells. Phenotypic changes were similar, regardless of which viral isolate was used.

first passage a further increase in detached cells could be observed within the culture of inoculated HepG2 cells. Cells reached confluence after an additional week and further sampling, together with a second culture passage, was performed. As the amount of detached cells in the supernatant was significant at the time of harvest, the supernatant was centrifuged, the pellet washed three times with PBS and resuspended in 200µl of PBS. These samples were referred to as the 'apoptotic fraction'. HepG2 cells were further incubated but cell viability was below 30% (as assessed by trypan blue exclusion). Culture medium was changed repeatedly and the cells slowly recovered. Cells were passaged when confluent and sampling continued over 6-7 weeks.

6.5.1.2 PCR analyses of HepG2 cells, following cell culture passages

Supernatant fluids collected during the one week exposure period were all positive for KSHV DNA as illustrated in figure 6.5. However, following cell propagation, KSHV specific DNA could only be found in a limited number of samples, in particular in the supernatant samples derived from cells originally inoculated with a high number of KSHV particles (inoculum: 10^8, day 14) and in the cell lysate from cells inoculated with $5x10^7$ KSHV particle. In contrast, other supernatant fluids were negative, as were all third wash samples and cell lysates. This has been observed in the 3-day study described above, where cells could only be infected infrequently. However, due to the increased amount of detached cells, the 'apoptotic fraction' was also tested. Interestingly, although cell lysates were negative, the collected detached cells (apoptotic fraction, AP) were positive throughout, suggesting that KSHV may infect

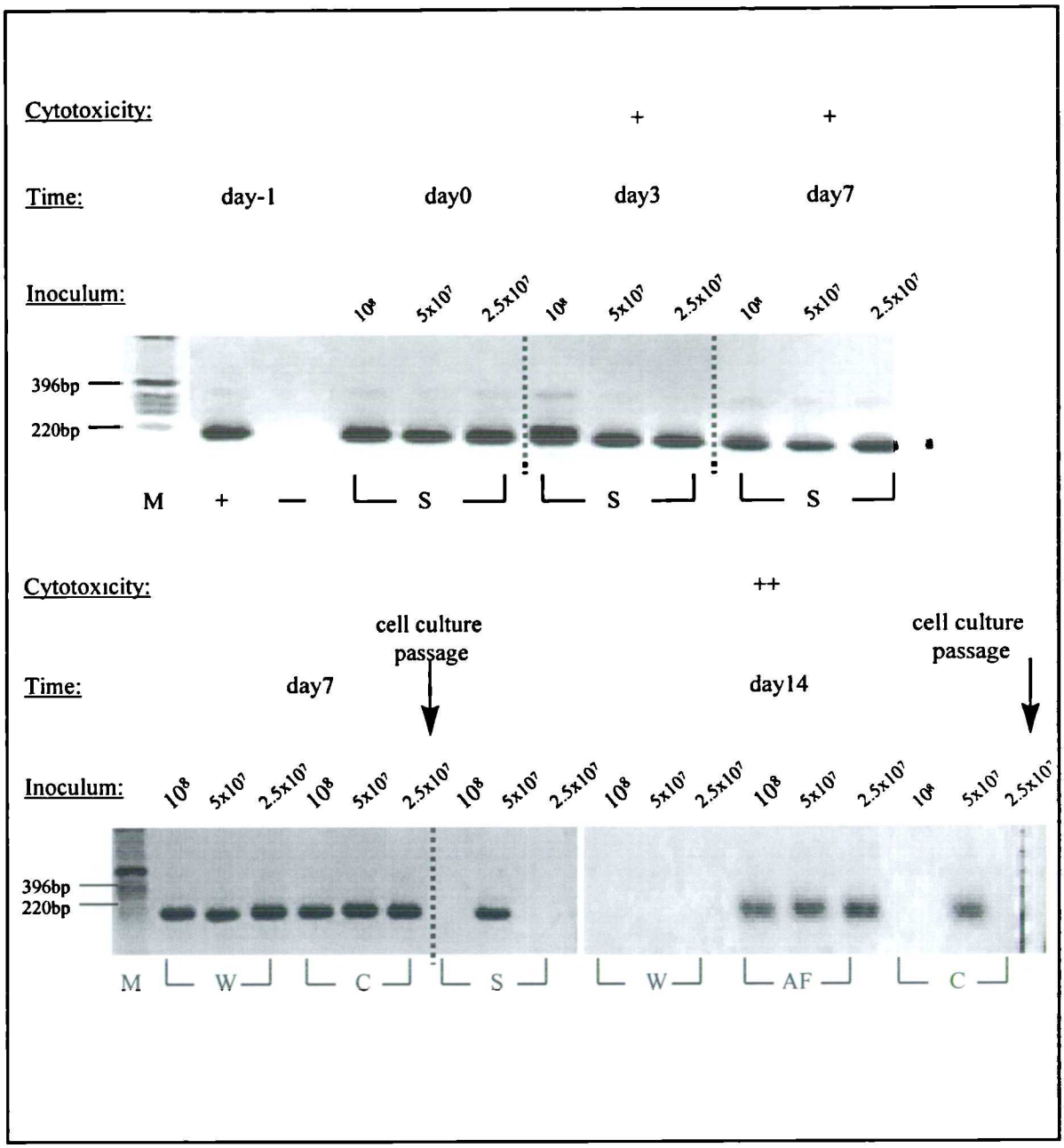


Figure 6.5: Infection of HepG2 cells by KSHV derived from BCP-1 cells. Cells were exposed to the BCP-1 supernatant for 7 days. Samples were collected as indicated by numbers above the lane (time), total DNA extracted and tested for viral DNA by PCR. Amount of virus contained in the inoculum together with the degree of cytotoxicity are mentioned on top. S: cell culture supernatant fluid; W: third wash sample; C: Cell lysate sample; AF: apoptotic fraction; (+) positive control (BCP-1 supernatant); (-) negative control (non-infected HepG2 cell lysate).

cells and induce cell detachment. This would explain the increase in detached cells observed throughout the experiment.

Unfortunately, PCR analyses showed that the KSHV genome was lost in culture after cells have been split a second time (data not shown). To be certain that no KSHV DNA was present, we maintained each HepG2 cell culture for two further passages and monitored supernatant, third wash together with cell lysate by PCR. Gel electrophoresis of the PCR products confirmed the previous result, no KSHV amplification products could be visualized (data not shown).

Overall, it appears that HepG2 cells are 'semi-permissive' for KSHV after an extended period of exposure. Cytotoxic effects could be observed in HepG2 cell cultures exposed to KSHV-containing supernatant. However, infection over serial passages could not be demonstrated, suggesting that this HepG2 infection was abortive. Whether the apoptotic effect seen was due to the presence of KSHV remains to be determined, though the presence of KSHV in the AF, when absent from the cell formation makes this probable.

6.5.2 KSHV derived from the SY cell line ($KSHV^+/EBV^+$)

6.5.2.1 Phenotypic changes in inoculated HepG2 cells

HepG2 cells were inoculated with cell free supernatant derived from SY cells under similar conditions as detailed above. Sampling was performed immediately after inoculation, 1 day PI, 3 days PI, 7 days PI, 14 days PI, 28 days PI, 35 days PI, 42 days PI, etc.. Initially the cytotoxic effect was not as prominent as described for the HepG2 cells inoculated with BCP-1 derived inoculum, even though cell growth was slightly

reduced. No significant phenotypic difference between inoculated cells and the mock-infected cells could be observed. Only after cells were propagated could an apoptotic phenotype and an increasing amount of detached cells be found in the supernatant. Similar to the studies described above, HepG2 cells continued to detoriate and cell culture medium had to be changed repeatedly. Following the second cell culture passage cells recovered slowly and after 2 weeks of incubation a sufficient number of cells were available to continue sampling. Unfortunately, cells remained prone to cytotoxicity and maintaining healthy cultures was difficult. Cells were propagated for three additional passages and finally recovered completely as the frequency of detached cells declined.

6.5.2.2 PCR analyses of SY exposed HepG2 cells

The amplification patterns of the samples taken during the 6 weeks of incubation are shown in figure 6.6. KSHV specific DNA was found in all samples prior to the first cell culture passage, including supernatant, third wash and cell lysate. Analogous to infection of HepG2 cells with BCP-1 material, certain samples remained positive after the first propagation. However, in contrast to BCP-1-infected HepG2 cells, strong PCR signals were detected for both cell lysate samples and the apoptotic fraction. Cells that were initially exposed to the highest and the lowest amount of viral particles were consistently positive for KSHV DNA (inoculum: 10^8; $2{,}5x10^7$; day 14).

A slightly different amplification pattern could be observed after cells were split a second time. Supernatant fluid and the third wash were negative for KSHV infection, whereas HepG2 cell samples and the corresponding apoptotic fraction of cells

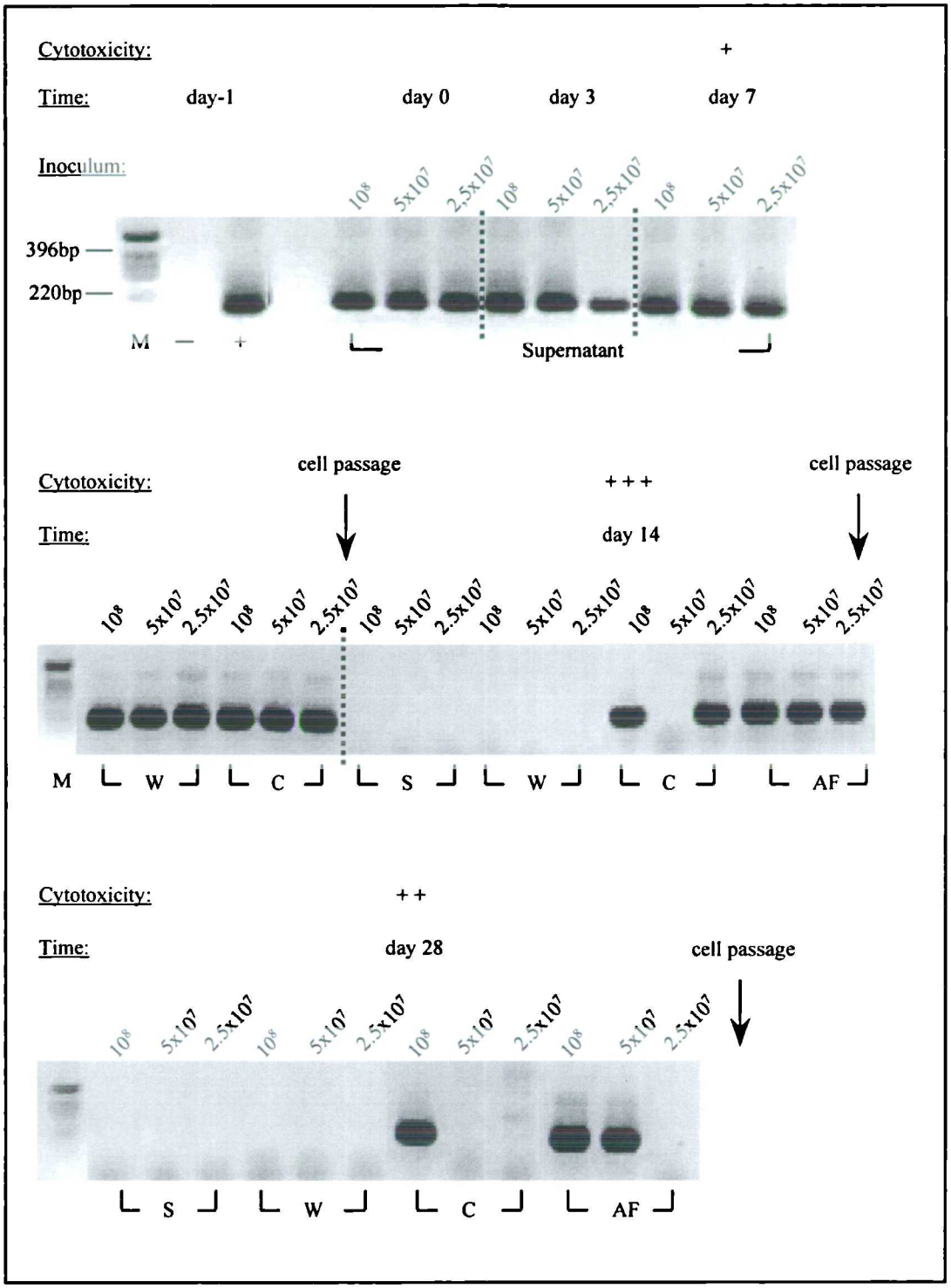


Figure 6.6: Long term infection of HepG2 cells inoculated with KSHV derived from the B cell line SY. Cells were inoculated for 7 days as described in the text. Samples were collected over a period of 6-7 weeks and analysed by nested PCR. Time of cell culture passages are indicated by arrows. S: cell culture supernatant; W: third wash sample; C: Cell lysate sample; AF: apoptotic fraction; (+) positive control (SY supernatant); (-) negative control (non-infected HepG2 cells). Degree of cytotoxicity is mentioned on top of each lane together with the amount of virus present in the inoculum. Mock-infected HepG2 cells were negative throughout the experiments (data not shown).

inoculated with 10^8 genome copies still contained viral DNA. However, this data could only be obtained when HepG2 cells were originally incubated with the highest viral titer. Cells being exposed to dilutions of infectious supernatant only showed positive amplification when analysing the apoptotic fraction (inoculum: $5x10^7$), or the signal was lost completely (inoculum: $2,5x10^7$).

The next culture passage was delayed (28 days PI), due to elevated cell detachment. Unfortunately, viral DNA could not be found after additional culture passage, all samples taken at later time points being negative. This may have been due to the poor condition of the infected HepG2 cells. The loss of PCR amplification maybe related to the immense cell death observed for each dilution. Surprisingly, KSHV DNA disappeared and the cells recovered, indicating that the virus may play a role in cell death within infected HepG2 cells. In comparison to the BCP-1 infection study, viral DNA derived from SY supernatant was maintained more efficiently, as judged by the intensity of the band, than from singly infected B cells. As SY cell supernatant contained both EBV and KSHV it appears that EBV may influence the ability of KSHV to infect HepG2 cells. All samples obtained from mock-infected HepG2 cells were negative for KSHV DNA (data not shown).

6.5.3 Sodium butyrate treatment of KSHV-infected HepG2 cells

Studies described above only investigated the presence of KSHV DNA, but do not address whether HepG2 cells are able to support viral replication or the production of viral particles. Attempts were therefore made to establish whether HepG2 cells support KSHV replication when induced with sodium butyrate. Sodium butyrate has

repeatedly been shown to selectively induce KSHV replication in B cell lines co-infected with EBV (Miller *et al.* 1997; Renne *et al.* 1996). HepG2 cells were infected with KSHV as described above and sodium butyrate added to the culture medium following the second cell culture passage at a final concentration of 10μM. Cells were incubated for 48 hours and samples taken. Productive infection was expected to result in the presence of KSHV within the supernatant or an increase in PCR signal intensity of HepG2 cell lysate. However, no increase in viral DNA within the supernatant or cell lysate was detected (data not shown). In fact the infected HepG2 cells remained susceptible to apoptosis and the viral genome was either lost or a weak signal remained (data not shown). Overall, sodium butyrate treatment did not change the amplification pattern compared to non-induced control cultures. This data suggests that KSHV infection of HepG2 cells is likely to be inefficient and production of viral progeny is either extremely low or non-existent. However, the lack of viral replication may be due to the loss of the viral genome. Further induction studies have not been performed as infection of HepG2 cells with KSHV was inconsistent.

To conclude, the data presented indicates that HepG2 cells do have a susceptibility to KSHV derived from SY cells. Furthermore during the experiment an association between KSHV and an incraesed number of dead cells, compared to the non-infected cell culture, could be observed. To begin with, viral DNA was found in viable cells, whereas further on in the experiment the PCR signal was lost from healthy cells and predominantly associated with detached, apoptotic cells, indicating that KSHV may induce cytotoxicity. More detailed studies are necessary to confirm this observation.

6.6 Summary and discussion

The successful propagation of KSHV in cell lines other than the established B cells derived from PEL patients has been limited. Certain epithelial cell lines, in particular primary or immortalized dermal microvascular endothelial cells (DMVEC) and certain other primary endothelial cells have been shown to support a long term productive infection (Panyutich *et al.* 1998; Moses *et al.* 1999; Flore *et al.* 1998). These infected cell lines developed a spindle shape, resembling KS spindle cells in vivo (Moses *et al.* 1999). Although KSHV sequences could be found in other cells with different origin, including lymphoid tissue, PBMCs, semen and saliva, studies show that in vitro infection is mostly abortive and the genome is lost upon passage (Renne *et al.* 1998; Foreman *et al.* 1997; Vieira *et al.* 1997; Moore *et al.* 1996). A similar observation was made in the present study using the hepatoma cell line HepG2. This cell line was 'semi-permissive' for KSHV infection, in that cells supported the entry of the virus.

This suggests that HepG2 cells may possibly express a surface receptor that facilitates KSHV entry. Since the viral genome was lost in serial passages and viral replication could not be induced by chemical stimuli, it is likely that this infection was abortive. Quantification of the PCR signal indicated that a minority of HepG2 cells harbored the viral genome. This corresponds to data obtained with other primary cell lines, where less than 10% of KSHV-challenged cells could be infected (Flore *et al.* 1998; Panyutich *et al.* 1998; Renne *et al.* 1998).

6.6.1 Viral replication may induce apoptosis

Interestingly, infection correlated with cell death of HepG2 cells and hence the maintenance of KSHV infected cells in culture was difficult. KSHV induced apoptosis has been reported previously for the embryonic kidney cell line 293 when using viral isolates derived from KS lesions (Foreman *et al.* 1997; Friborg *et al.* 1998). In contrast, virus derived from a PEL cell line was not able to induce apoptosis in 293 cells and serial passages were ineffective (Friborg *et al.* 1998). Further sequence analysis identified strain variability among different KSHV isolates, particularly between those derived from KS lesions and B cell lymphomas (Zong *et al.* 1997; Friborg *et al.* 1998). Assuming that apoptosis may be the result of viral replication, KSHV-infected HepG2 cells were further induced with sodium butyrate. However, the production of virus could not be confirmed as judged by the negative PCR results of supernatant and cell lysate samples. Cytotoxicity could be ascribed to a number of factors other than the virus itself, such as culture conditions or senescence. Interestingly, HepG2 cells have been reported to support KSHV replication, when analysed in an RT-PCR based assay specific for a spliced mRNA (U. Ayliffe, personal communication; Renne *et al.* 1998). This suggests that the lack of expression described here may be due to methodological problems, such as growth conditions, the differentiation state of HepG2 cells or the insensitivity of the detection method. To detect low levels of replication the analysis of RNA rather than DNA may be advantageous. Further studies investigating the gene expression pattern using monoclonal antibodies against lytic viral proteins would address these questions.

6.6.2 The presence of EBV

In our study the presence of the gammaherpesvirus EBV appeared to promote maintenance of KSHV in HepG2 cells. Viral DNA was found more consistently in HepG2 cells that had been inoculated with supernatant from the dually infected cell line SY ($KSHV^+/EBV^+$). In contrast, inoculation with KSHV only resulted only in transient infection and the genome was lost after the first cell culture passage. The fact that KSHV could be more readily detected when EBV was present is interesting. Recent data examining KSHV *de novo* infections of PBMC, revealed that for a stable infection, EBV is necessary (Kilche *et al.* 1998). B cells infected with both viruses, KSHV and EBV appear to possess a growth advantage, suggesting that one virus may exhibit transforming properties whereas the other provides growth-promoting co-factors. This model is further supported by the fact that most PEL derived B cell lines are co infected with KSHV and EBV. It has been suggested that EBV can promote the replication of other viruses in vitro, e.g. the replication of the Hepatitis C virus (HCV) (Sugawara *et al.* 1999). EBV has been frequently detected in association with HCV in patients with heptatocellular carcinoma. Studies of these two viruses suggests that the EBV latent nuclear antigen EBNA1 may to be responsible for the support of HCV replication. EBNA1 is a DNA binding protein with affinity to certain sequence motifs within the origin of latent viral DNA. It functions in the maintenance and replication of the EBV episome but may also be involved in regulating the transcription of foreign genes (Rawlins *et al.* 1985; Reisman and Sugden, 1986; Liebowitz, 1993). The interaction of EBV and HCV has also been confirmed in HepG2 cells and it is possible that EBV functions as a helper virus, similar to adeno-associated virus, which requires co-infection with a second virus (Adenovirus or herpesvirus) for productive

infection (Berns and Giraud, 1996). HepG2 cells are permissive for EBV, although the primary EBV receptor, CD21, is not expressed. A restricted pattern of latent viral gene transcription can be observed, including the proteins EBNA1, EBER and LMP2A (Imai *et al.* 1998). In the present study the EBV latent expression pattern was not analysed, but one can assume that a similar scenario will apply in HepG2 cells inoculated with SY supernatant. Therefore, it could be possible that KSHV replication is similarly influenced by EBV. KSHV encodes analogs of EBV nuclear antigens, including the latency associated nuclear antigen, LNA-1. This protein associates with the mitotic chromosome and may have a similar function to the EBV nuclear antigens. However, studies have shown that in B cells co-infected with KSHV and EBV, EBNA1 and LNA-1 do not colocalize, implying that the proteins bind to different nuclear domains (Szekely *et al.* 1998). EBNA1 may transactivate certain KSHV genes or assist in the maintenance of the KSHV genome in culture. If EBNA1 does enhance KSHV replication the increase in apoptotic cells could be explained and analyses of protein expression soon after inoculation should be positive. However KSHV-infection of the EBV-positive Burkitt's B-lymphoma cell line Raji was unsuccessful. The KSHV genome was lost with serial passages and productive lytic replication was limited (Moore *et al.* 1996). It hence cannot be excluded that EBV is non-essential for KSHV infection in HepG2 cells. Studies, in which HepG2 cells were infected with EBV and subsequently exposed to supernatant derived from BCP-1 cells (KSHV+/EBV-) are important to assess whether EBV influences KSHV infection in HepG2 cells. More detailed studies of the KSHV expression pattern in HepG2 cells at

different times during infection are important to determine whether HepG2 cells do indeed allow viral replication.

In summary, the human hepatoma cell line HepG2 is susceptible to KSHV infection, but how supportive this cell line is for viral replication in the long term remains to be established. Unfortunately, during the KSHV infection study HepG2 cells could not be maintained in culture for a prolonged period of time. Thus, no long term culture system could be established. Whether the virus itself was responsible for the increased number of cells deteriorating remains to be determined.

The findings that KSHV can infect this hepatoma cell line may have considerable relevance to the *in vivo* situation. There is a possibility that viral infection is not only associated with epithelial cells of the skin, but also with cells of the liver. However, there is so far no evidence *in vivo* that KSHV may infect the liver.

7. Development of a KSHV quantitative assay

7.1 Introduction

Human herpesviruses can both cause primary disease and reactivate during immunosuppression, resulting in severe or life-threatening illnesses, such as shingles, CMV retinitis and lymphoproliferative disorders. In KS lesions and primary effusion lymphomas the majority of cells are latently infected as judged by *in situ* studies. However, there is little known about primary infection of KSHV and the molecular mechanism of KSHV reactivation. In PEL cells lytic replication can be induced using chemical stimuli. There are several virally encoded proteins that have been demonstrated to exhibit transactivating properties, but the factors which may trigger viral reactivation *in vivo* remain to be established. To study KSHV pathogenesis, and to monitor the influence of antiviral therapy on viral replication, an easy and rapid quantification assay would be highly desirable. So far several approaches have been described, mainly based on PCR procedures (Bai *et al.* 1997; Gessain *et al.* 1997; Lock *et al.* 1997). Some are 'semi-quantitative', others are based on the co-amplification of unknown quantitative target sequence with a known amount of a competitive template (Hodinka, 1998; Lock *et al.* 1997). The assay described in this chapter is based on the nested PCR primers described in chapter 6 and is designed to be performed in a 96 well plate. Amplicons are detected by complementary probes that produce a quantifiable chemiluminescent signal, analogous to an EIA. To determine the viral copy number external standards are included in the assay that encompass a $5\log_{10}$

dynamic range. A variety of samples, including tissue culture material and blood samples, have been tested and the assay enabled their successful quantification.

7.2 The design of the quantification assay

The PCR-based quantification assay design used here was originally developed for the quantitative detection of Hepatitis C RNA (Whitby and Garson, 1995). Figure 7.1 illustrates a schematic diagram of this assay, called qPCR (for quantitative PCR) or ELONA (for Enzyme Linked Oligonucleotide Assay). Its principal can be applied to any infectious diseases where a quantitative result is required. The assay can be divided into three stages, the extraction of nucleic acid, the specific amplification of target sequences and ,finally, detection. Figure 7.1 illustrates the detection process which includes capture of biotinylated PCR product onto streptavidin coated plates (1), subsequent denaturing of double stranded DNA (2), hybridisation of an alkaline phosphatase oligonucleotide probe (3) and finally an enzymatic reaction which produces a quantifiable chemiluminescent signal (4). A detailed description of each step is given below.

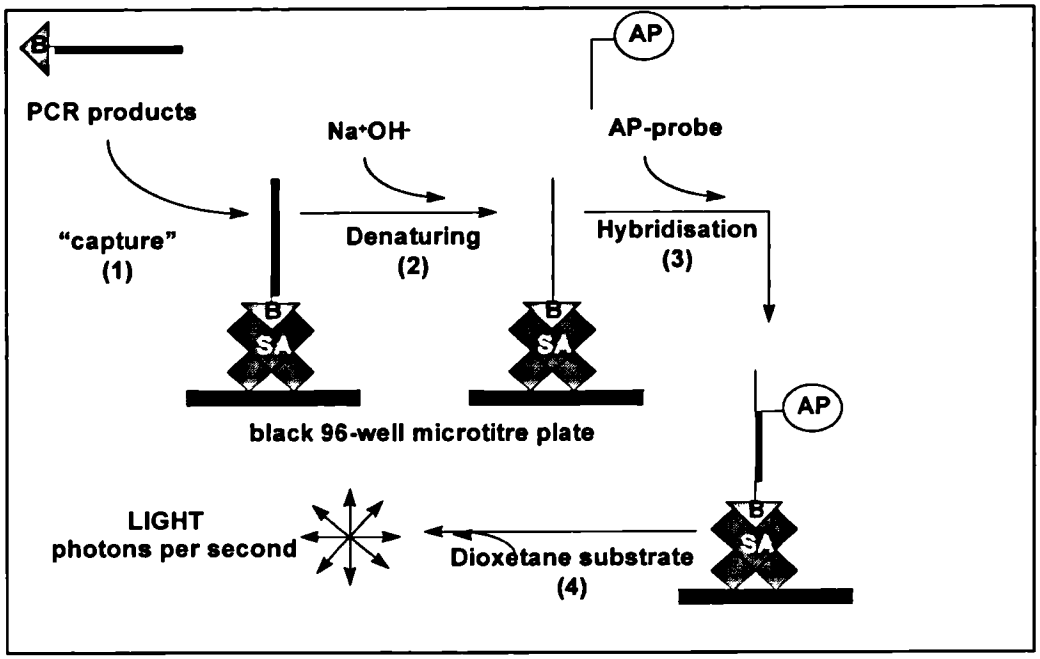


Figure 7.1: Schematic picture of the KSHV quantification assay. Kindly provided by Dr K Whitby. B: Biotin; SA: Streptavidin; AP: alkaline phophatase

7.2.1 Extraction of total DNA

KSHV DNA was extracted from each sample using a commercially available extraction kit (Qiagen, QIAamp blood mini kit). It is important for the successful amplification of the target sequence to remove potential PCR inhibitors, such as heparin or hemoglobin. In order to monitor extraction efficiency a known amount of control DNA (derived from a different virus) may be added to each sample prior to DNA purification. Herpesvirus saimiri or the murine herpesvirus 68, both closely related to KSHV, would be an ideal control target. Oligonucleotide primers have to be designed that co-amplify both viruses, KSHV and the second gammaherpesvirus, together with subsequent detection procedure to differentiate

between the two viral species. This method to assess extraction efficiency has been successfully applied for a hCMV quantitative assay, using murine CMV as an internal control (Dr W Preiser, personal communication). However, for the quantification assay presented here this internal control has been omitted, but could be included with a minor modification of the amplification reactions.

7.2.2 The amplification process

Following DNA extraction, viral DNA is amplified in a single round PCR. To enable immobilization of the amplification product the 5' end of one of the oligonucleotide primers has been biotinylated, allowing capture onto a streptavidin coated plate. The amplification primers, together with the alkaline phosphatase oligonucleotide probe, were carefully selected from sequence alignments of all known KSHV. To cover a diversity of samples of different origin, it is essential to choose amplification target sequences that are well conserved. The KSHV open reading frame 26 appears to be an ideal choice since its gene product, the viral minor capsid protein, is well-conserved among the gammaherpesviruses, displaying 60% amino acid identity (Zong *et al.* 1997). Furthermore, ORF26 has been the subject of several epidemiological and evolutionary studies, showing that sequence variability in this region is low, approximately 2% (Moore and Chang, 1995; Zong *et al.* 1997). Based on the first available viral sequences of KSHV, soon after its identification, a nested PCR was designed by M. Howard in 1997 and was available for this study. In addition, a hybridisation probe was designed as part of a multiplex assay for the detection of

all human herpesviruses (M Howard, PhD thesis, 1999; for further details see chapter 2 and 6 of this thesis). However, since more sequence data was available to us the oligonucleotide primers were re-evaluated to ascertain whether they matched the majority of samples. Following BLAST searches, all available sequence data was aligned and examined for possible mis-matches within the primer regions. Unfortunately, the published data are restricted to just part of ORF26, giving alignments covering only the inner primer sites. In consequence, further analyses focused on this region, as mis-matches that may affect primer binding of the outer set could not be excluded.

As highlighted by the sequence alignment in figure 7.2, no sequence variation could be observed between the majority of samples, including sequences derived from patients with classical KS (CKS), AIDS-associated KS (AKS), endemic KS (AFR) and PEL cell culture derived material. In contrast, the original alkaline phosphatase probe showed one variation, highlighted in red. Since this single base pair polymorphism is located within a 14bp primer, it is unlikely to affect hybridization reactions. However, it has to be taken in consideration and less stringent hybridisation conditions may be required.

In summary, the inner set of oligonucleotides are located within a conserved region and were selected for the amplification of KSHV specific DNA as part of the quantitative assay.

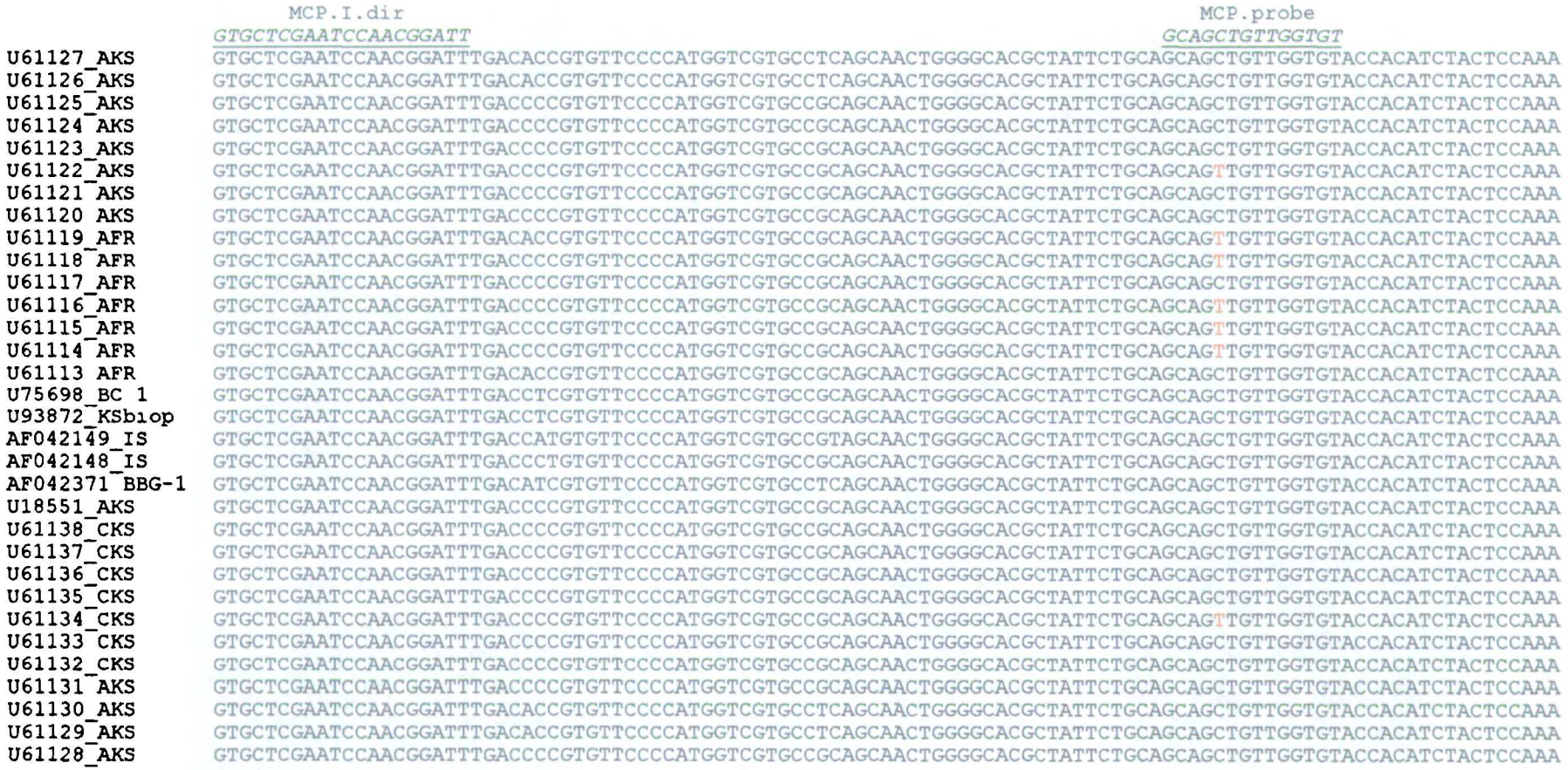


Figure 7.2: Sequence alignment of a region within KSHV ORF26 (nucleotide 47308 to 47479 with reference to Russo *et al.* 1996). Following Blast search, all sequences available were aligned and examined for miss matches within the primer annealing regions. Miss matches are highlighted in red; names of oligonucleotide primers are written above the sequences. AKS, AIDS-KS; CKS, classical KS; AFR, African KS; BC-1, PEL cell line; KSbiop, KS biopsy; IS, isolate derived from a patient without HIV or HTLV infection, BBG-1, BBG-1 cell line.

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                                                                                                MCP.I.RC
                                                                                                ATATACCACCAATGTGTCAT
U61127_AKS      ATATCGGCCGGGGCCCCGGGTGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61126_AKS      ATATCGGCCGGGGCCCCGGGTGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61125_AKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61124_AKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61123_AKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61122_AKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61121_AKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61120_AKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61119_AFR      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61118_AFR      ATATCGGCCGGGGCCCCGGATGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61117_AFR      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61116_AFR      ATATCGGCCGGGGCCCCGGATGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61115_AFR      ATATCGGCCGGGGCCCCGGATGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61114_AFR      ATATCGGCCGGGGCCCCGGATGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61113_AFR      ATATCGGCCGGGGCCCCGGGTGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U75698_BC-1     ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U93872_KSbiop   ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
AF042149_is     ATATCGGCCGGGGCCCCGGATGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
AF042148_is     ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
AF042371_BBG-1  ATATCGGCCGGGGCCCCGGGTGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U18551_AKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61138_CKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61137_CKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61136_CKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61135_CKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61134_CKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61133_CKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61132_CKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61131_AKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61130_AKS      ATATCGGCCGGGGCCCCGGGTGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61129_AKS      ATATCGGCCGGGGCCCCGGGTGATGTCAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
U61128_AKS      ATATCGGCCGGGGCCCCGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATG
```

Figure 7.2: continued

7.2.3 The detection process

Once amplicons were immobilized on streptavidin coated microtiter plates, the DNA was denatured with sodium hydroxide (150 mM) and hybridised with an alkaline phosphatase conjugated oligonucleotide probe. Following addition of a dioxetane-based chemiluminescent substrate, Lumiphos (Lumigen Inc.), the intensity of emitted light was measured by a luminometer (TopCount, Canberra Packard Inc.). To allow quantification, calibration standards containing a known amount of KSHV DNA (defined by limit dilution PCR) were included in each amplification set. These calibrators were serially diluted, as described below, to cover a range of quantities. The standards were amplified in separate tubes (external controls) and allowed the establishment of a calibration curve against which the signal obtained from each sample was plotted.

7.2.4 Data analysis

Optimal quantification is achieved when the viral DNA is exponentially amplified. Under such conditions the concentration of the amplicon is proportional to the amount of target sequence originally added and therefore allows accurate quantification. Briefly, each sample has been analysed in duplicate and the mean calculated. In the case of poor duplicates (< 20% difference) quantification was repeated. Using the data produced by the calibration standards a calibration curve ('curve of best fit') was generated. Following simple algebraic equations (interpolation), the viral copy number of the test samples could be determined.

7.2.5 The calibration standards

Controls (standards) can be either external or internal, depending whether they are amplified in the same reaction tube as the sample (internal) or separately (external). In the case of the KSHV quantitative assay, external standards were prepared from a BCP-1 cell culture. As described before, this cell line is singly infected with KSHV and hence is an ideal calibration standard for the quantification assay. Cell culture supernatant was collected and total DNA was extracted from 200µl aliquots. Total DNA was resuspended in 50µl of nuclease-free water. The number of viral genomes within these samples was determined by limiting dilution PCR as detailed in chapter 2. Serial dilutions were prepared at the limit dilution end point, each set comprising 10 tubes, to obtain a sufficient frequency of positive and negative amplifications. Reactions were separated by gel electrophoresis, negatives scored and the copy number calculated. In figure 7.3 the last two dilutions, 10^{-6} and 10^{-7} respectively, showing amplification are shown. From these assays we concluded that the selected sample contained a high amount of viral DNA, 105400 copies per microliter. For the quantification assay seven standards are prepared, a true negative (no DNA), a second negative that contains 'more or less 0 copies' (10^{-7} dilution, 0.1 copies) and four controls that cover a range of copy numbers. This dilution series was freshly prepared for each test to avoid possible template DNA degradation. Aliquots of the limit dilution quantified total DNA were stored at -20°C and one sample used at a time. The standards described here were used throughout the study and copy numbers were 10540; 1054; 105; 10; 1; 0.1 and water as a negative control respectively.

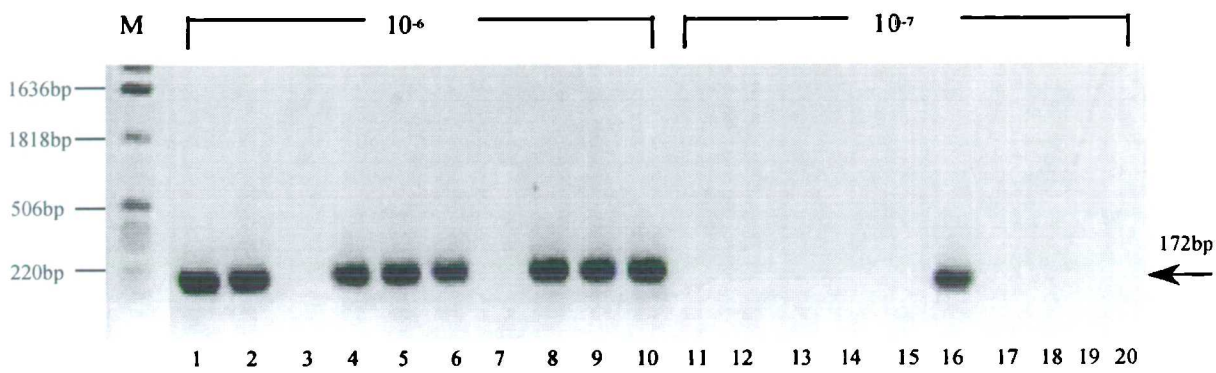


Figure 7.3: Endpoint dilution PCR of BCP-1 supernatant (Poisson distribution). BCP-1 supernatant (200µl) was collected and total DNA extracted. Total DNA was diluted 1/100 followed by 10 fold dilutions. Viral DNA was amplified by nested PCR and analysed by 1% agarose gel electrophoresis (for detailed method see chapter 2).
Lane 1-10: 10^{-6} dilution
Lane 11-20: 10^{-7} dilution
M: 1kb DNA marker (Life Technologies)

7.3 Optimization of the quantitative assay

The PCR conditions and the subsequent detection conditions were optimized using the calibration standards described above.

7.3.1 The ELONA-PCR

The quantification assay presented in this chapter is based on a 'single round' PCR (see section 7.2.2 for further details). To avoid non-specific binding of the probe during the detection step, the amplification of satellite products has to be minimised. Reaction conditions were optimized using the standards as target DNA. The polymerase TaqGold (Perkin Elmer) was used, allowing a 'hot start PCR' (12min at 95°C). Primer concentrations were assessed and amplicons examined on a 1% agarose gel. Figure 7.4 illustrates DNA fragments amplified with (A) 200nM and (B) 500nM final concentration of each primer. The extracted DNA was serially diluted in water and used for a single round PCR using the PCR conditions described for the nested PCR, except for the initial denaturing step which was extended to 12min at 95°C. As evident from figure 7.4, the higher concentration of primers resulted in a more prominent amplification, in particular at lower template concentrations (lane 9 and 10). All subsequent reactions were therefore performed using 500nM (f.c.) of oligonucleotide primers.

7.3.2 The ELONA

The amplification products were captured onto a microtiter plate and different concentrations of the detection probe evaluated. Briefly, calibration curves were similar, though higher concentration of the probe resulted in increased signal

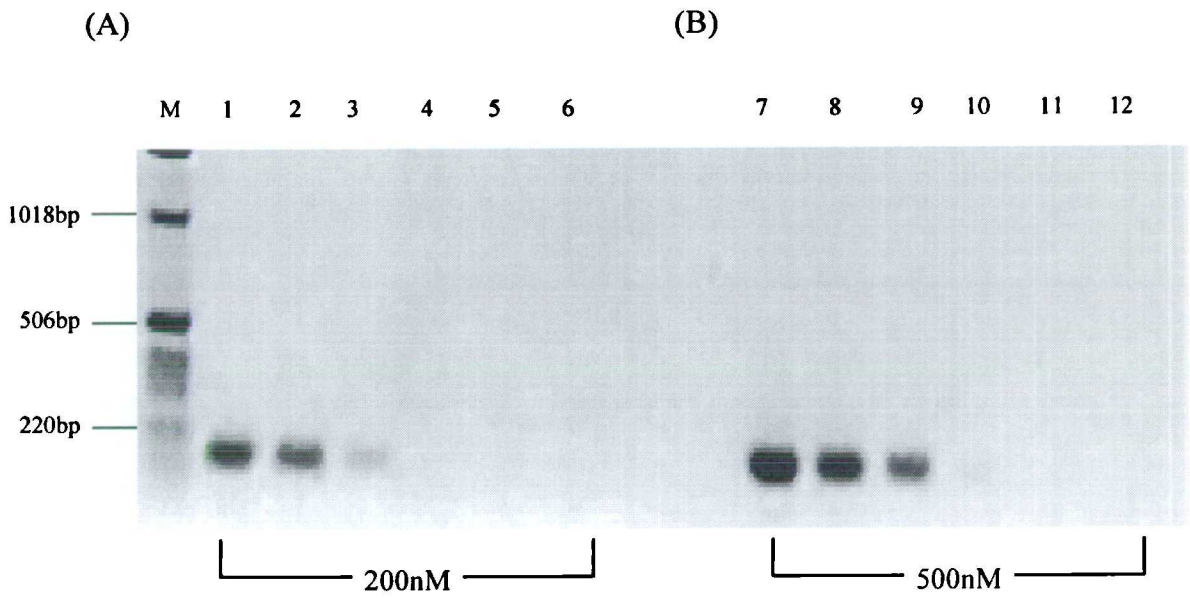


Figure 7.4: Single round PCR of KSHV calibration standards. DNA derived from BCP-1 supernatant was serially diluted as detailed in the text. Following a PCR for 35 cycles amplicons were separated on a 1% agarose gel. Dilution factors are indicated at the bottom of each lane; M indicates the 1kb ladder.

(A) PCR using 200nM f.c. oligonucleotide primers

Lane 1: 10^{-2}
Lane 2: 10^{-3}
Lane 3: 10^{-4}
Lane 4: 10^{-5}
Lane 5: 10^{-6}
Lane 6: 10^{-7}

(B) PCR using 500nM f.c. oligonucleotide primers

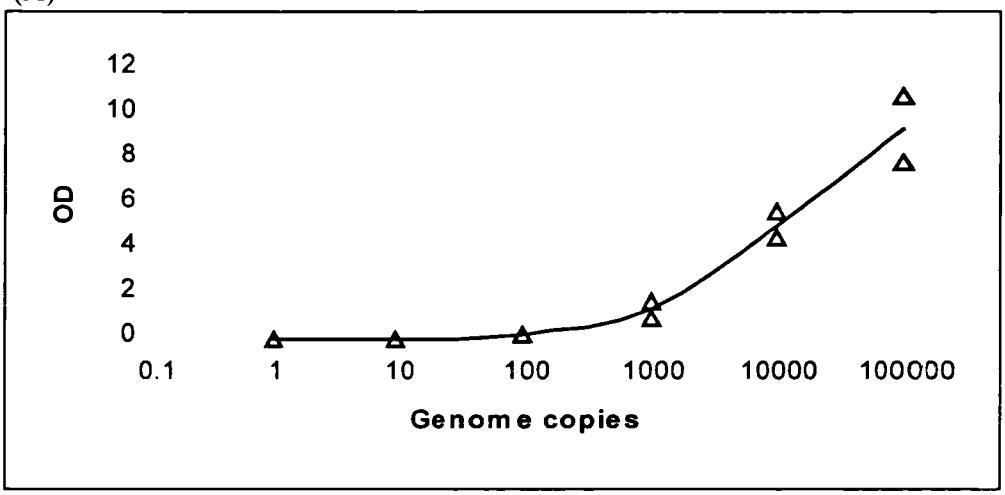
Lane 7: 10^{-2}
Lane 8: 10^{-3}
Lane 9: 10^{-4}
Lane 10: 10^{-5}
Lane 11: 10^{-6}
Lane 12: 10^{-7}

intensities for negative controls (data not shown). Consequently, a dilution which produced little background but a high signal for positive samples was used throughout the study, 1 in 1800 (2.7nM f.c).

7.3.3 The optimal combination of PCR and ELONA

Following PCR and probe optimization, cycle conditions were assessed. The aim was to establish a system that identified low viral copy numbers, rather than high copy numbers. For this, the most important distinction was that between the 'likely negative' (<1 copy) sample and the last positive sample (1 copy). As a consequence 35 and 38 cycles of amplification were compared. The assay was performed as detailed in chapter 2 and data analysed using the computer software Genesis. Both calibration curves are shown in figure 7.5. Triangles represent each duplicate. With the increase in cycle numbers the assay changed its dynamic range. More cycles of amplification result in a more accurate 'bottom end', but higher amounts of input DNA enter the plateau phase, making it difficult to distinguish between higher viral load samples. In contrast, fewer cycles produced a reversed situation, favoring discrimination between higher copy numbers. For research purposes, studying KSHV reactivation or drug susceptibility, an assay quantifying low copy numbers appeared to be more valuable. Thus, in the following series of experiments all samples were amplified in a 38 cycle PCR, using 500mM (f.c.) of oligonucleotides primers.

(A)



(B)

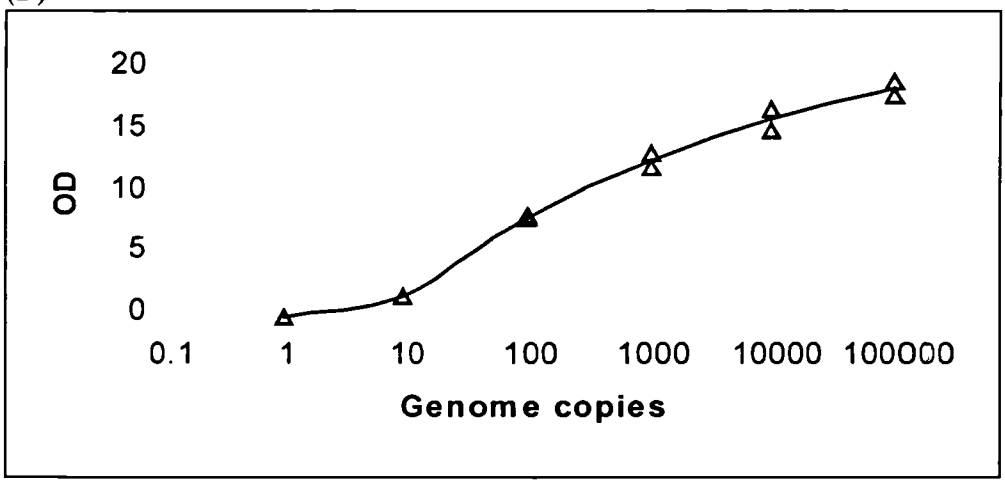


Figure 7.5: Calibration curves after optimization of the PCR cycle number. KSHV calibration standards were amplified by non-nested PCR and genome copy numbers determined by ELONA as detailed in the text. Calibration samples were analysed in duplicates. Each value is indicated by a triangle.
(A) 35 cycles of amplification
(B) 38 cycles of amplification

7.4 Quantification of a variety of samples, including whole blood, plasma, and tissue culture material

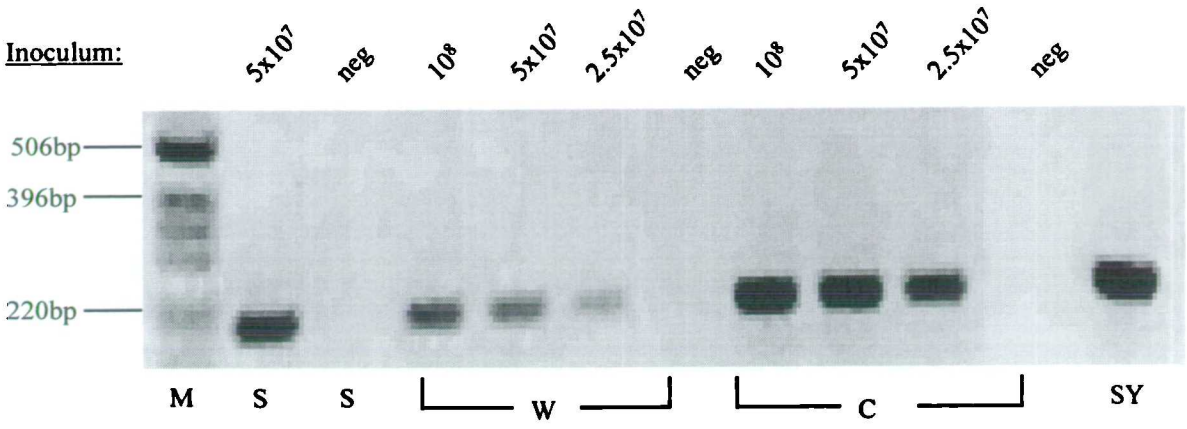
Once the viral DNA standards had been optimized and the assay could be consistently reproduced, samples that were positive and negative by nested PCR were tested. These included tissue culture samples obtained as part of the HepG2 infection study (see chapter 6) and EDTA-blood, plasma and serum samples from HIV-infected individuals with and without KS were tested.

The EDTA-blood was separated by centrifugation and the plasma collected and replaced by an equal volume of PBS (reconstituted blood). All serum samples were obtained as frozen aliquots from UCH diagnostic service. Total DNA was purified from 200µl aliquots of all samples using a commercial extraction kit (see chapter 2). The DNA was diluted in 50µl of nuclease-free water and 1µl (tissue culture material) or 10µl were analysed by quantitative PCR.

7.4.1 Tissue culture material

Chapter 6 describes the infection of HepG2 cells with different amounts of KSHV inoculum. Samples were collected over a period of time and analysed by nested PCR for the presence of viral DNA. These samples were expected to contain a high amount of virus as judged by the intensity of the nested PCR amplicon (see chapter 6). Several samples were selected, including different HepG2 cells inoculated with different dilutions of the viral DNA, and re-tested using the quantification assay. Copy numbers within each sample are demonstrated in figure 7.6. together with a gel picture of the single round PCR products.

(A)



(B)

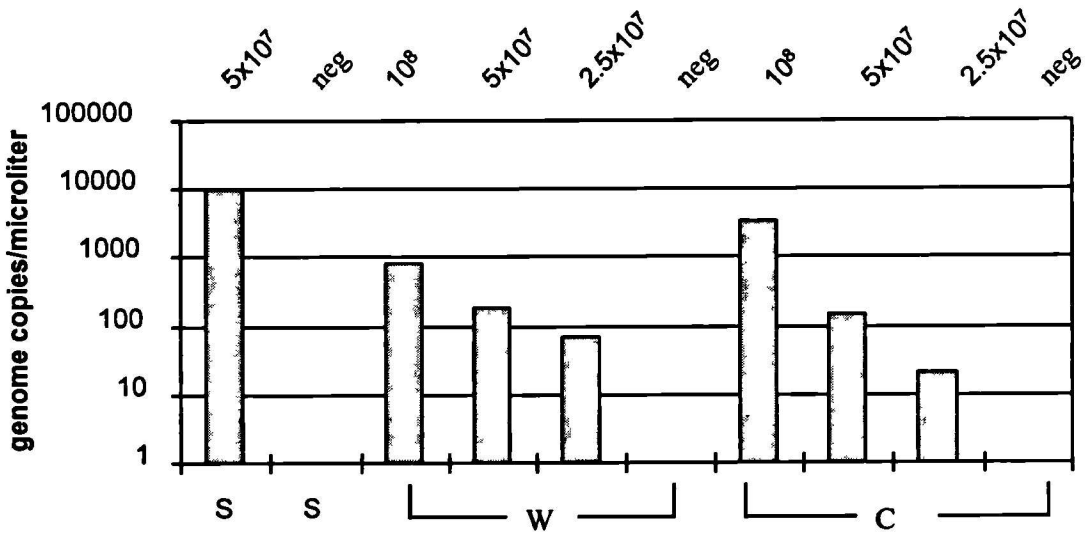


Figure 7.6: KSHV quantitative analyses of HepG2 cell culture samples. Cell culture supernatant from HepG2 cells inoculated with KSHV were collected 7 days post-Infection as described in chapter 6. Total DNA was extracted and tested by qPCR. Each number represents the mean of 2 duplicate determinants. Undiluted SY supernatant (SY) was also analysed, but the genome copy number was out of range for this assay, therefore only the amplification product is shown.

(A) 1% agarose gel showing the 172bp amplicons.

(B) Calculated copy numbers per microliter of extracted DNA, detected in tissue culture samples derived from KSHV infected HepG2 cell cultures.

S: supernatant; W: wash; C: cell lysate; neg: negative; M: 1kb marker. Numbers on top of each lane refer to the initial KSHV inoculum.

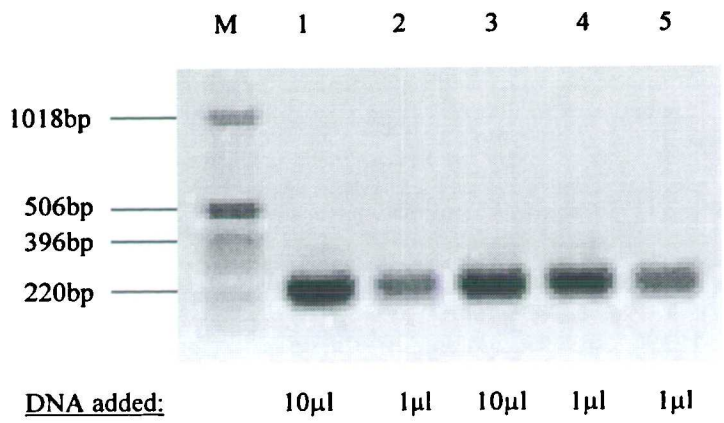
The figure (panel B) of the viral copy numbers shows a decrease in viral DNA across each set of samples, concomitant with the band intensity shown in panel A. This confirms that HepG2 cells were exposed to serial dilutions of the viral inoculum. The number of copies within the inoculum is indicated above each lane in panel A. This demonstrates that this assay is able to quantify viral load in tissue culture samples and that the amount of viral genomes calculated roughly corresponds to the band intensity of the PCR. In summary, the ELONA produced reliable results and may support further infection studies.

Samples with higher loads of virus, e.g. supernatant derived from SY cells were also tested and produced a signal that was outside the dynamic range of this assay (data not shown). This result is unsurprising as the calibration standards were based on viral DNA derived from tissue culture material. To quantify SY supernatant or other high titer samples a dilution series would be necessary.

7.4.2 Whole blood, separated plasma and serum

In a similar assay to that described above, blood samples obtained from three KS positive patients were tested by both nested and quantitative PCR (qPCR). Serum samples from the same patients, but taken at a different time point than the blood samples, were also analysed by qPCR. Following DNA extraction from plasma, whole blood and serum samples, KSHV viral load was quantified. The genome copy numbers obtained are shown in figure 7.7, together with a photograph of the nested PCR products.

(A)



(B)

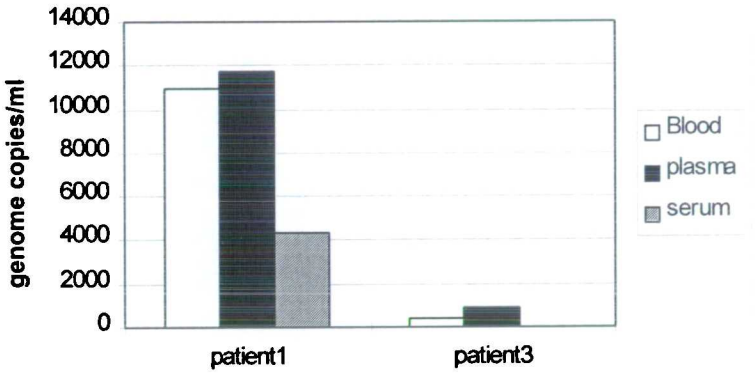


Figure 7.7: KSHV viral load in blood samples obtained from individuals with AIDS-associated KS. Total DNA was extracted from reconstituted blood (Blood), EDTA-plasma and serum samples. One or ten microliters of total DNA were analysed by nested and quantitative PCR. Each number represents the mean of duplicate determinants.
(A) Nested PCR of patient sample 1, 1 and 10µl are indicated; M: 1kb DNA marker. Lane 1 and 2: EDTA-blood; lane 3 and 4: EDTA-plasma; lane 5: positive control (BCP-1 supernatant).
(B) Quantification of blood samples from patient 1 and 3.

Unfortunately, only two sets of samples were positive for KSHV by nested and quantitative PCR, regardless of the DNA input amount. Although KS was diagnosed in patient 2, KSHV could not be detected by nested PCR in any sample. KSHV viral load within the nested PCR positive samples (patients 1 and 3) was subsequently determined and the data indicated that KSHV was more abundant in plasma (panel B, black bar) and whole blood (white bar) than in serum (shaded bar). This corresponds to reports for other viruses, e.g. HIV RNA, where the genome cannot be detected as efficiently in serum than in plasma or whole blood (Semple *et al.* 1991). Furthermore, these serum samples had been freeze/thawed several times and hence may not represent the initial viral load after sampling. However, this study does demonstrate that KSHV can be found in blood, in particular in plasma, and to a limited amount in serum samples, from individuals with KS. Further studies, including a greater number of samples and plasma/serum pairs taken at the same time point will be necessary to investigate the association between plasma and serum viral load and are ongoing.

7.4.3 Plasma samples and serum samples from a KS cohort

Our initial series of experiments showed that KSHV viral load can be estimated using plasma samples. We were interested in testing samples to examine the association of viral load and antibody titer within KS patients as part of a collaboration with Dr C Boshoff (Molecular Pathology). To do this, further plasma and corresponding serum samples were tested. A total of 50 pairs of plasma and serum samples were selected from a cohort of HIV-infected individuals with unknown KS status to investigate viral load within both types of

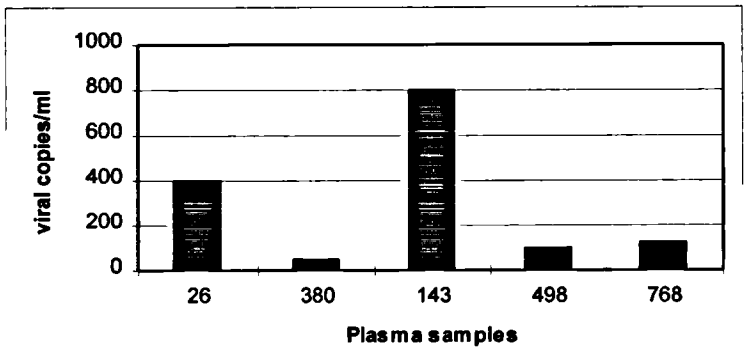
samples. The majority of this cohort included mainly homosexual men with less than 5% HIV-infected women. These samples had been stored for some time at -80°C and may have been freeze/thawed a number of times.

Epidemiological studies showed that KSHV is more prevalent in HIV-infected individuals than in the general population, therefore a proportion of these samples was expected to be positive. Since KS had not been diagnosed within these patients at the time of sampling, the viral titer was expected to be low, when detectable at all. Total DNA was extracted from each sample as described before, 10µl applied to a single round PCR and KSHV viral load quantified. Firstly, the plasma samples were tested by qPCR for KSHV DNA as described above. Subsequently, the corresponding serum samples for the KSHV positive plasma samples were also quantified. These data are summarized in figure 7.8.

Only 5 plasma samples (10%) were positive for KSHV DNA, with less than 1000 copies per ml of plasma. This data agrees with previous epidemiological studies where the virus has been described to be present in 5-30% of HIV-infected gay men. In contrast, corresponding serum samples did not reflect a similar viral frequency, KSHV being found in only one sample, containing ~50 copies per ml. This indicates that the detection of KSHV is relatively unreliable in serum samples, though the virus can be identified in plasma samples. Viral particles present in serum samples are more often subject to degradation, viral genomes seem to be more stable when stored as plasma, in particular EDTA-plasma (P. Grant; personal communication). It is possible that the EDTA present in plasma samples acts as a nucleic acid 'preservative' by chelating Mg^{2+} from the sample

and hence inactivating nucleases. From this experiments we may conclude that studies of KSHV incidence and quantity using only serum samples could not be performed. However, the KSHV quantification assay presented in this chapter can be successfully used to investigate in more detail epidemiological as well as other aspects that are relevant for KSHV pathogenesis.

(A)



(B)

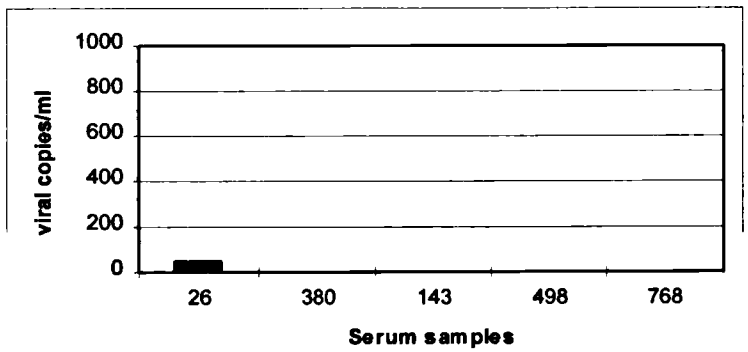


Figure 7.8: KSHV viral load in plasma and corresponding serum samples from HIV-positive individuals without a history of KS. A total of 50 patients was analysed by qPCR as detailed in the text. Samples were obtained from a cohort of HIV-infected individuals, including homosexual men and less than 5% women.
(A) Positive plasma samples and viral loads
(B) Serum samples corresponding to positive plasma samples and viral loads

7.5 Summary and Discussion

Serological findings indicate that KSHV infection is rare in the general population but significantly higher in certain risk groups, including areas where KS is endemic, HIV-infected individuals (in particular homosexual men) and immunosuppressed patients. Little is yet known about the primary infection or the exact route of transmission of KSHV. Epidemiological studies imply sexual transmission, as HIV-infected homosexual men appear to be at high risk for developing KS. In contrast, in areas with endemic KS many children are infected indicating infection during childhood. Detailed analyses of KSHV transmission and primary as well as secondary infections have been restricted to serological methods or qualitative PCR. So far there is limited data available that investigated viral load and disease association. The development of a reliable quantification assay would greatly support such studies. For example in HIV research quantitative analyses of viral load has been widely applied and hence allowed further insight into the pathogenesis of the virus, in particular demonstrating the association of plasma viral load with disease stage (Ho *et al.* 1989; Semple *et al.* 1991). The detection of high levels of HIV-1 RNA in PBMCs or plasma correlated with disease stage. Lower titers of HIV-1 could be found in asymptomatic patients, whereas symptomatic patients contained higher viral loads. Similarly, in EBV-associated disease EBV DNA could be detected more frequently and at higher levels during the acute phase of the illness (Yamamoto *et al.* 1995). Recently, KSHV viral load has been determined in PBMCs from HIV-infected individuals. The study revealed that high amounts of KSHV are a predictor of KS development (Min and Katzenstein, 1999). Similarly, in organ transplant patients,

increased levels of KSHV in peripheral blood lymphocytes have been associated with the development of KS (Mendez *et al.* 1999). There are several clinically relevant aspects that would greatly benefit from the development of a KSHV quantitative assay, such as monitoring disease progression as well as anti-retroviral therapy and assessing the risk of perinatal transmission. The latter may be more relevant for areas where KS is endemic as there is evidence that KS may be transmitted from mother to child (Angeloni *et al.* 1998).

The KSHV qPCR presented in this chapter is designed to analyse a variety of different sample material within a short period of time. This allows studies into viral load in wide population cohorts. The 96-well microtiter format supports the simultaneous analyses of a very large number of samples, making it attractive for epidemiological research purposes. The assay has been tested successfully using different sample material, including tissue culture samples, whole blood, human plasma and serum. Testing matching plasma and serum samples from HIV-infected individuals, we noted that KSHV could be detected more consistently in plasma samples and whole blood. This has been previously reported when testing PBMCs and plasma from KS patients, PCR detection demonstrated that KSHV sequences found in PBMCs correlated with the level found in plasma (Harrington *et al.* 1996). In our study plasma was the most successful PCR template which led us to test a small cohort of samples obtained from HIV-infected patients without KS. The cohort included both women (<4%) and gay men. Overall, 5/50 patients were identified to contain KSHV in their plasma. This correlates with the sero-prevalence being elevated within this risk group (20-25%) (Gao *et al.* 1996;

Simpson *et al.* 1996; Chatlynne and Ablashi, 1999; Martin *et al.* 1998). In particular homosexual men show high rates of anti-KSHV antibodies in contrast to HIV-infected women, where approximately 4% contain serum antibodies (Kedes *et al.* 1997; Lennette *et al.* 1996). In our study only 10% of the EDTA-plasma samples were positive for KSHV, which may have been due to the selection of men and women for this study. KSHV appears to be sexually transmitted and studies showed that the number of homosexual intercourse correlates with an elevated risk for KS in Europe (Melbye *et al.* 1998). Since there was no detailed data about the sexual behavior of this cohort available we cannot exclude that this aspect may have an influence on the low detection rate reported in this chapter.

However, when we analysed the corresponding serum samples, the virus could not be found in most of the samples, indicating that serum is a poor sample source for KSHV viral load testing. Several studies examining the stability of other viruses, such as HIV-1 or HCMV have shown that the viral genome can be more frequently found in cell-free plasma than in serum samples (Holodniy *et al.* 1995; Ginocchio *et al.* 1997).

Several localization studies indicated that KSHV is harbored in PBMCs and testing of whole blood may be indicated (LaDuca *et al.* 1998; Rizzieri *et al.* 1997); Harrington *et al.* 1996). We detected KSHV with almost the same frequency in whole blood and plasma, concluding that both sample source can be used in viral load studies. However, larger population-based studies would be required to identify the test material of choice and to introduce this assay into routine diagnostic testing.

Clinically a KSHV quantification assay has applications for the diagnosis and management of individuals with KS as well as individuals that are HIV positive without KS. Studies showed that the detection of KSHV in the blood of HIV-infected individuals is a predictor for the development of KS (Kennedy *et al.* 1998; Gao *et al.* 1996; Moore *et al.* 1996b; Whitby *et al.* 1995). Early detection would allow efficient treatment and the possibility to prevent the development of KS. This would be desirable, as KS is the most common neoplasm in AIDS patients. Furthermore, using this assay KSHV viral load can be monitored in response to highly active anti-retroviral therapy (HAART) and may help in identifying effective treatment procedures.

Besides clinical applications viral load analyses can support investigating the transmission route of KSHV. As mentioned before (see chapter 1), in Africa KSHV can be found with a high frequency in children and therefore may be transmitted from mother to child (Angeloni *et al.* 1998). Serological studies using serum sample pairs from mothers and children suggest that high antibody titer correlate with high viral load and therefore indicate transmission from mother to child (Bourboulia *et al.* 1998). However, whether high titer of KSHV antibodies correlate with high viral load is contradictory (Campbell and Borok, 1999) and requires more detailed studies using a reliable quantification assay.

However, the data presented in this chapter indicates that this quantification assay is able to determine KSHV viral load within a variety of samples. Further studies comparing this qPCR assay to other existing quantification assays, such as limit dilution/Poisson distribution, semi-quantitative or TaqMan based assays are

necessary to compare the assays' sensitivity. Finally the introduction of an internal control has to be considered to monitor DNA extraction efficiency. Nevertheless, this type of assay has been successfully applied in the past to quantify other viral infections, and from the data presented here it is most likely to be beneficial for KSHV research as well as diagnostic purposes. The very broad dynamic range, 4 to 5 $\log_{10}$, is able to encompass a wide viral load range and will assist in examining different viraemia levels within KS patients, risk groups, general populations or individuals with KS undergoing anti-viral therapy. Using this assay a number of studies addressing epidemiological and molecular aspects of KSHV, can be performed.

8. Final Conclusions

The newly identified herpesvirus KSHV (HHV8) is associated with Kaposi's sarcoma and the lymphoproliferative disorders primary effusion lymphoma and multicentric Castleman's disease. KSHV is unique within the herpesviral family as it encodes an exceptional large number of proteins with homology to cell cycle regulatory proteins and inflammatory cytokines. Some of these cellular proteins as well as their viral counterparts, have been shown to exhibit oncogenic properties and hence may play an important role in tumorgenesis. Before KSHV was identified studies showed KS to be highly associated with inflammatory cytokines, which support the growth of the spindle cells characteristic of a KS lesion (Ensoli *et al.*1992; Ensoli *et al.* 1989). These findings, and the repertoire of KSHV encoded cytokines, suggest that KSHV is at least a co-factor in the pathogenesis of KS. Furthermore, epidemiological studies support the hypothesis that KSHV is the most likely causative agent of KS. Within the general population the sero-prevalence is low, compared to the sero-prevalence within risk groups (Mediterranean Europe, Africa, HIV-infected homo- and bisexual men and immunosuppressed individuals) where KSHV can be found more frequently (Gao *et al.*1996; Kedes *et al.*1996; Simpson *et al.*1996; Calabro *et al.*1998; Lennette *et al.*1996; Chandran *et al.*1998). In addition to epidemiological studies, several investigations have concentrated on the in-vitro characterization of KSHV ORFs that may exhibit oncogenic properties. Several viral proteins have been demonstrated to influence tumor suppressor pathways (ORF72; v-cyc) as well as other cell signaling pathways (ORF74; vGPCR), inhibit apoptosis (ORF16; vbcl-2) or induce cell transformation (ORFK1). By acting in concert and forming a

complex cascade, these viral proteins could play a key role in the development of uncontrolled cell growth. However to date the data is limited to recombinant expression of isolated genes and has not yet been confirmed by *in-vitro* viral infection studies. In addition, little is known about the viral entry mechanism and the envelope glycoproteins of KSHV. KSHV glycoprotein B (gB) has been recently described and analysis of the intracellular processing and expression of this viral protein has revealed similar structural features to previously characterized herpesviral gB, in particular EBV gp110. However, this study also showed that there are functional differences. Although KSHV gB and EBV gp110 can form hybrids, KSHV gB was unable to complement gp110-negative EBV mutants (Pertel *et al.* 1998). Besides KSHV gB, no other surface glycoprotein has been analysed so far. Studies into virus-host cell interactions have been limited, which may have been due to the lack of a *de novo* infection system for KSHV. From PCR and in-situ analysis of a variety of different cells it is clear that the KS-typical spindle cells harbor KSHV as do PBMCs and monocytes (Boshoff *et al.*1995; Blasig *et al.*1997). However, which cell in particular is targeted by the virus is unknown. Recently, endothelial cell lines have been successfully infected with KSHV long-term, indicating the presence of receptor molecules (Moses et al. 1999). Several aspects of KSHV have been addressed in this project, all contributing in their own terms to a better understanding of viral pathogenesis. The main objective of this project was to characterize certain KSHV envelope glycoproteins and possibly examine cell surface interactions with host cells. In addition a target cell line has been identified that is semi-permissive for KSHV

which implies that the virus exhibits a relatively broad cell tropism. Finally, an assay has been established that allows the rapid quantification of KSHV and may contribute to epidemiological as well as molecular questions.

8.1 KSHV ORF22/gH and ORF47/gL, two tricky proteins

Soon after the identification of KSHV, a small subset of the viral genome was available, including the open reading frames encoding the tegument protein, thymidine kinase, glycoprotein H and the major and minor capsid proteins. To study possible viral interactions with the host cell ORF22, predicted to encode glycoprotein H, appeared to be a reasonable choice. Considering the well described association of gH with gL in other herpesviruses, the KSHV gL homologue was included in the study. Herpesviral gH and gL mediate cell to cell fusion and are described to contribute to viral penetration into the host cell. The exact mechanism is unknown, although gH is believed to play the key role in fusion events, whereas gL appears as a chaperone that influences gH processing and transport to the cell surface. Thus, this protein family would be ideal to examine viral interactions with the cell surface. However, the recombinant expression of both proteins was unexpectedly complicated and made receptor studies impossible within the given time frame. However, basic characteristics of KSHV glycoprotein L could be identified. The data presented here indicates that KSHV gL has a similar pattern of intracellular localization as the gL homologs encoded by EBV or HCMV. The recombinant protein was predominantly found in the cytoplasm, most likely retained in the ER and the nuclear membrane.

Interestingly, unlike its homologs, there is evidence that KSHV gL expressed in the epithelial cell line 293HEK and in the fibroblast cell line Cos7 may induce cell to cell fusion. Overall, data from dual labeling studies and western blot analyses indicate that multinucleated cells contain an elevated amount of soluble and processed KSHV gL. This observation further indicates that gL appears to induce fusion when recombinantly expressed in 293HEK or Cos7 cells. Although more definitive localization studies are required to confirm our observations, the current data suggests that KSHV gL itself contributes to cellular fusion, contradictory to the general opinion that gH is the major factor in the fusion process. However, we cannot exclude that the formation of multinucleated cells was due to over expression of the protein rather than a fusogenic epitope. In addition, the lack of a direct detection system for recombinant KSHV ORF22/gH made detailed studies difficult. We were unable to distinguish gH in co-expression studies and therefore syncytia formation in dually transfected cells may have been induced by gL alone, instead of the gH/gL complex, as seen in other herpesviruses. For further studies the use of monoclonal antibodies or different protein tags are advisable to examine the intracellular distribution of ORF22/gH. Distinct surface expression in cells co-transfected with KSHV gH and gL could not be observed, only minor changes in the localization pattern of the fluorescent signal were detected. Further studies analysing the co-localization of Golgi and gL expression in co-transfected cells were unsuccessful. However, these particular studies were limited as the expression level was low and the altered intracellular distribution pattern itself was restricted to a minority of cells. As mentioned above several studies will be

required to confirm the data presented, in particular the expression of KSHV gH has yet to be studied in detail.

As the butyrate induction studies revealed higher expression, it is likely that both proteins exhibit toxicity. Perhaps a vector system where the protein production can be controlled will assist in studying recombinant expression in more detail, as possible toxic effects by the recombinant protein could be prevented. The production of monoclonal antibodies would also simplify the detection process and will allow more detailed analyses of the recombinant protein, including epitope mapping. In summary, these basic studies show that the expression pattern of both proteins is complex and that different vector systems may be useful to further analyse KSHV gH and gL. To conclude, although the examination of possible viral-host cell interactions could not be completed, KSHV gL appears to have some influence in the fusion process, which suggests that more investigations should concentrate on this protein's properties.

Apart from gH/gL a third protein may be required that allows authentic complex formation. With the description of a third viral component associating with the gH/gL complex in EBV and HCMV, the possibility that the recombinant expression of KSHV gH and gL similarly depends on another viral protein cannot be ruled out and should be addressed in future. Monoclonal antibodies specific for gH or gL, when available, can assist in precipitating the gH/gL complex from PEL cell lines and will give insights into the processing of these two viral glycoproteins.

8.2 KSHV, EBV and the liver?

B cell lines derived from primary effusion lymphomas are mostly co infected with KSHV and EBV. Analyses of the expression pattern of EBV revealed that these cells express EBNA1 and to a lower level LMP1, whereas essential latent antigens which are usually expressed in lymphoblastoid cell lines could not be detected (Horenstein *et al.* 1997). This indicates that KSHV has some influence on EBV expression either directly, or indirectly by inducing cellular proteins which then regulate the EBV latency expression pattern. As part of this project the hepatoma cell line HepG2 was inoculated with KSHV, susceptibility to KSHV could be increased when using supernatant from an EBV/KSHV dually infected B cell line. HepG2 cells are the first epithelial cell line that can be dually infected with KSHV and EBV. KSHV infection was associated with cytotoxicity, indicating possible viral replication. Unfortunately, KSHV replication could not be demonstrated and a long-term culture system could not be established, possibly as a result of enhanced cytotoxicity. Apoptosis has been observed in B cell lines infected with KSHV that have been established from patients with PEL. These cell lines tend to exhibit a necrotic phenotype in culture unless they are co-cultured with a feeder-layer cell line, such as HEL cells. This indicates that the HEL cells produce factor(s) that influence the ability of KSHV to induce apoptosis. The nature of this factor remains to be established. KSHV encodes two proteins that have been demonstrated to inhibit apoptosis, vbcl-1 and vFLIP (see chapter 1). However, both proteins are expressed during latency, although vbcl-2 only at low levels (Sarid *et al.*1997), and may under certain circumstances, such as *in vitro* cell culture conditions or cell-type specific conditions, induce rather then inhibit

apoptosis. Identifying the factor(s) produced by the feeder-layer that influences PEL cell growth could be important in the understanding of the association between KSHV and apoptosis as well as in facilitating the growth of these particular cell lines and possibly other culture systems.

Several factors have been demonstrated to influence KSHV replication, such as chemical stimuli and viral proteins. Recently, it has been shown that infection of a KSHV infected PEL cell line with HIV resulted in the induction of lytic KSHV replication and enhanced transmission of KSHV to uninfected target cells (293 cells) (Varthakavi *et al.*1999). Furthermore a soluble factor has been shown to stimulate KSHV lytic replication in this system, but its identity remains to be defined. Early reports examining the association of KSHV and HIV showed that the HIV transactivator protein, Tat , can activate KSHV replication in PBMCs from KS individuals (Harrington *et al.*1997). Overall, these data indicate that KSHV is able to interact with other viruses. However, the mechanism behind this interaction is far from understood. It is most likely that KSHV and EBV also interact, since co-infected B cells have been found regularly in PEL. Interestingly, KSHV encodes proteins that are similar to the cellular proteins induced after EBV infection, e.g. v-cyc and cellular D-type cyclin. This may indicate a link between these two viruses, with EBV regulating the expression of KSHV genes or the reverse. Our transient culture system may assist in studying these questions further using an epithelial cell line rather than B cells. Although further studies are required to explore the EBV and KSHV expression pattern, HepG2 cells have been clearly shown to be semi-permissive for KSHV, suggesting viral-host cell

interactions. So far KSHV has not been associated with liver-derived cells. Therefore to study the impact that KSHV infection may have on the HepG2 cell life cycle is interesting and may provide further insights into KSHV cell tropism. It is also possible that like EBV KSHV is associated with liver disease.

Although KSHV expression could not be demonstrated in HepG2 cells other procedures such as flow cytometry may detect low expression levels of different viral antigens. Also the analysis of cytokine levels within infected cultures may assist in characterizing KSHV infection in more detail.

In summary the HepG2 culture system allows studies into the interaction of KSHV and EBV as well as KSHV infection alone of a liver cell line with liver specific functions. Furthermore, HepG2 cells may also allow expression studies of KSHV gL and gH to investigate their functional properties and allow investigations into possible surface receptor(s) for KSHV.

8.3 The impact of a quantification assay

Since the identification of KSHV several serological assays have been developed that vary in their sensitivity (see chapter1). Initially we were interested to evaluate whether recombinant gH and gL could be included in such serological assay system. This idea was soon dismissed in favor of a quantification assay based on PCR amplification. The assay presented shares its design with other assays for the quantification of HIV, HCV and HCMV, indicating its ability to allow reliable quantification. The assay was modified to our purposes and several samples were tested to evaluate its ability to quantify KSHV. Using samples from a small cohort

of 50 patients infected with HIV we demonstrated that 10% of the patients exhibited detectable viral titers. This corresponds to the prevalence of KSHV found by others when tested individuals with HIV but without KS (Gao *et al.*1996; Kedes *et al.*1996; Martin *et al.*1998; Blackbourn *et al.* 1999). Though we only tested a limited number of samples, the data described here suggests that the test can be used for further studies into the biology of KSHV. Indeed, this assay is now being evaluated to assist studies of KSHV lytic replication and for inclusion in diagnostic testing.

Viral quantification can be applied both diagnostically and for research purposes. Measuring viral load within a patient can assist in monitoring disease progression or the impact of anti-viral therapy. In drug development this assay can be used to identify the potency of a particular drug. Furthermore, quantification can be used as a prognostic marker for disease development or for the detection of herpesviral reactivation. There are a number of examples where quantitative methods have been successfully applied and provided an insight into the association of viral replication and disease progression, in particular in HIV research (Berger *et al.*1997; Yamamoto *et al.*1995).

For KSHV specifically such an assay can support investigations of viral load and the development of KS. KS is the most common neoplasm in individuals with AIDS (Ensoli *et al.* 1991; Beral *et al.* 1990). Studies using qualitative PCR have shown that the presence of KSHV in HIV-infected individuals predicts KS development (Whitby *et al.* 1995). Furthermore, 20-35% of homosexual or bisexual men with AIDS are KSHV infected, compared to approximately 3% of

the general population (Gao *et al.* 1996; Kedes *et al.* 1996; Martin *et al.* 1998; Blackbourn *et al.* 1999; Simpson *et al.* 1996). It is therefore likely that KSHV is involved in KS pathogenesis. Monitoring patients at risk for KS would allow early responses as soon as an increase in viral load was detected and may achieve a decline in HIV-associated KS in the future.

Analysing KSHV viral replication, and the influence that various factors may have on it, can also be analysed conveniently with such an assay. A whole range of studies would benefit from this assay; viral gene expression can be reliably associated with viral load estimates or immunological responses such as antibody production, furthermore seroconversion and viral load can be examined.

In summary, we have established an assay that can address a variety of questions concerning KSHV reactivation, drug susceptibility, disease association and epidemiology.

To conclude KSHV is most likely to play an important role in the development of KS and PEL. Studying viral host-cell interactions offers the opportunity to identify new therapeutic targets that may lead to successful treatment or even vaccine development. KSHV is unique as it encodes for a large number of oncogenes, that allow to not only study viral pathogenesis but also to gain further information about the cell cycle regulation and angiogenesis. This thesis includes studies of several aspects of KSHV which can be the basis of more detailed characterizations of molecular entry mechanism and may help in identifying target cells. HepG2 cells appear to be susceptible to KSHV infection and may support

studies into the interaction of related viruses within one cell. Finally, an assay that is beneficial for both in depth epidemiological studies as well as diagnostic testing has been established as part of this thesis.

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Appendix I

Solutions and Buffer

10xTris-Saline

8.5% NaCl; 0.05% Tween-20

50x Tris Acetate EDTA (TAE), pH 8.1

242g/l Tris base; 57.1ml/l glacial acetic acid; 37.2g/l EDTA,

Tris-EDTA (TE)

1mM EDTA; 10mM Tris-Cl, pH 8

PBS

150mM NaCl; 15mM KH_2PO_4; 63mM $Na_2HPO_4xH_2O$; 26mM KCl; pH 7.2

Lysis buffer I (Triton-X-100 lysis buffer)

50mM Tris-HCl, pH 7.5; 140mM NaCl; 1% Triton-X-100, 1mM PMSF

Lysis buffer II (SDS-lysis buffer)

50mM Tris-HCl, pH6.8, 2% SDS

Flow cytometry

*P*BS*F*CS*A*zide

PBS; 1% FCS; 0.05% sodium azide

Western Blot analyses

Towbin Transfer Buffer

25mM Tris, 192mM glycine, 20% methanol, 0.1% SDS, pH 8.3

TBSTT

20mM Tris-Cl; 500mM NaCl; (pH 7.5); 0.2% (v/v) Tween-20;

0.3% (w/v) Triton-X-100

Tris-Marvel

20mM Tris-Cl; 500mM NaCl; (pH 7.5); 5% Marvel; 0.3% (v/v) Tween-20

Tris-Casein

20mM Tris-Cl; 500mM NaCl; (pH 7.5); 1% Casein; 0.3% (v/v) Tween-20

Tris-BSA

20mM Tris-Cl; 500mM NaCl; (pH 7.5); 2% BSA; 0.2% (v/v) Tween-20; 10% FCS; 5% Normal goat serum

Tris-NGS

20mM Tris-Cl; 500mM NaCl; (pH 7.5); 10% Marvel; 0.2% (v/v) Tween-20; 10% Normal goat serum; 5% FCS

EIA

EIA blocking buffer

6% BSA/0.05% Tween-20 in 20mM Tris/Azide

TMTFCS

4% low fat milk powder; 0.5% Tween-20; 20%; FCS in 1xTBS

TMFGT

20% Goat Serum, 4% Marvel, 0.5% Tween-20 in Tris buffered saline

PCR and Cloning

10xAnnealing buffer

200mM Tris-Cl, pH 8.4; 500mM KCl; 15mM $MgCl_2$

10xExpand PCR reaction buffer

500mM Tris-Cl, pH 9.2; 160mM $(NH_4)_2SO_4$; 22.5mM $MgCl_2$; 20%(v/v) DMSO; 1%(v/v) Tween-20

10xUniversal buffer

1M KOAc; 250mM Tris-Acetate, pH 7.6; 100mM MgOAc; 5mM β-mercaptoethanol; 100µg/ml BSA

Buffer H

50mM Tris-HCl, 10mM $Mgcl_2$, 100mM NaCl, 1mM Dithioerythritol, pH 7.5

10xLigase buffer

300mM Tris-Cl, pH 7.8; 100mM; $MgCl_2$; 100mM DTT; 5mM dATP

SDS-PAGE

10xPage buffer

25mM Tris-Cl; 192mM glycine; 0.1% SDS

Sample buffer (Laemmli buffer)

1.43M beta mercaptoethanol; 125mM Tris-Cl, pH 6.8; 20% glycerol; 6% SDS; 0.004% bromphenolblue

Coomassie blue solution

0.25g Coomassie brilliant blue; 45% methanol; 10% glacial acetic acid; 45% water

Destaining solution

10% acetic acid; 40% methanol; 50% water

Blue Cell Assay

Blue cell assay staining solution

3mM Potassiumhexacyano(II)ferrat; 3mM Potassiumhexacyano(III)ferrat; 1mM $MgCl_2$

X-Gal

0.5mg/µl X-Gal stock solution, diluted in staining solution to a final concentration of 5µg per µl

Agarose gel electrophoresis

1kb DNA Marker (Life Technologies)

20µl 1kb marker; diluted 200µl of TE and 80µl of loading dye

10xLoading dye

40% sucrose; 0.1% orange G

Sequencing

10xTrisBorateEDTA (TBE)

108 g/L Tris base, 55 g/L boric acid, 9.3 g/L EDTA, adjusted to pH 8.3

Quantitative Assay

10xTTA

50ml Tween-20, 10ml Azide in 1000ml of 1M Tris-Cl

Sample diluent

5% (v/v) Casein blocking buffer (Genesis, Ltd.); 0.5% (v/v) Tween-20 in PBS

Probe diluent

5% (v/v) Casein blocking buffer (Genesis, Ltd.); 0.5% (v/v) Tween-20 in 20xSSC

Bacterial growth medium and cell culture medium

Luria-Bertani (LB) (for 1000ml)

10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl

Ampicillin stock solution

100mg/ml ampicillin in water, stored at -20°C

Complete medium DMEM

Dulbecco's Modified Eagle's Medium, 10% FCS, 5mM L-glutamine, 100U/ml penecillin, 100μg/ml streptomycin, 0.075% sodium bicarbonate

Complete medium RPMI,

Dulbecco's Modified Eagle's Medium, 10% FCS, 5mM L-glutamine, 100U/ml penecillin, 100μg/ml streptomycin, 0.075% sodium bicarbonate

Selection medium, neomycin

Complete media supplemented with 500mg/ml geneticine

Selection medium, zeocin

Complete media supplemented with 200μg/ml zeocin

Freezing Medium

10% DMSO, 40% FCS, 50% DMEM/RPMI

PBS/EDTA

25mM EDTA in PBS

Appendix II

Figure II.1: Schematic map for pcDNA3 (Invitrogen). This vector was used for subcloning and sequencing of the tPA-glycoprotein fragment. Furthermore, this vector was used in generating pcDNA3.3p6. MCS: Multiple cloning site; BGHpolyA: polyadenylation signal and transcription termination sequences from the bovine growth hormone gene.

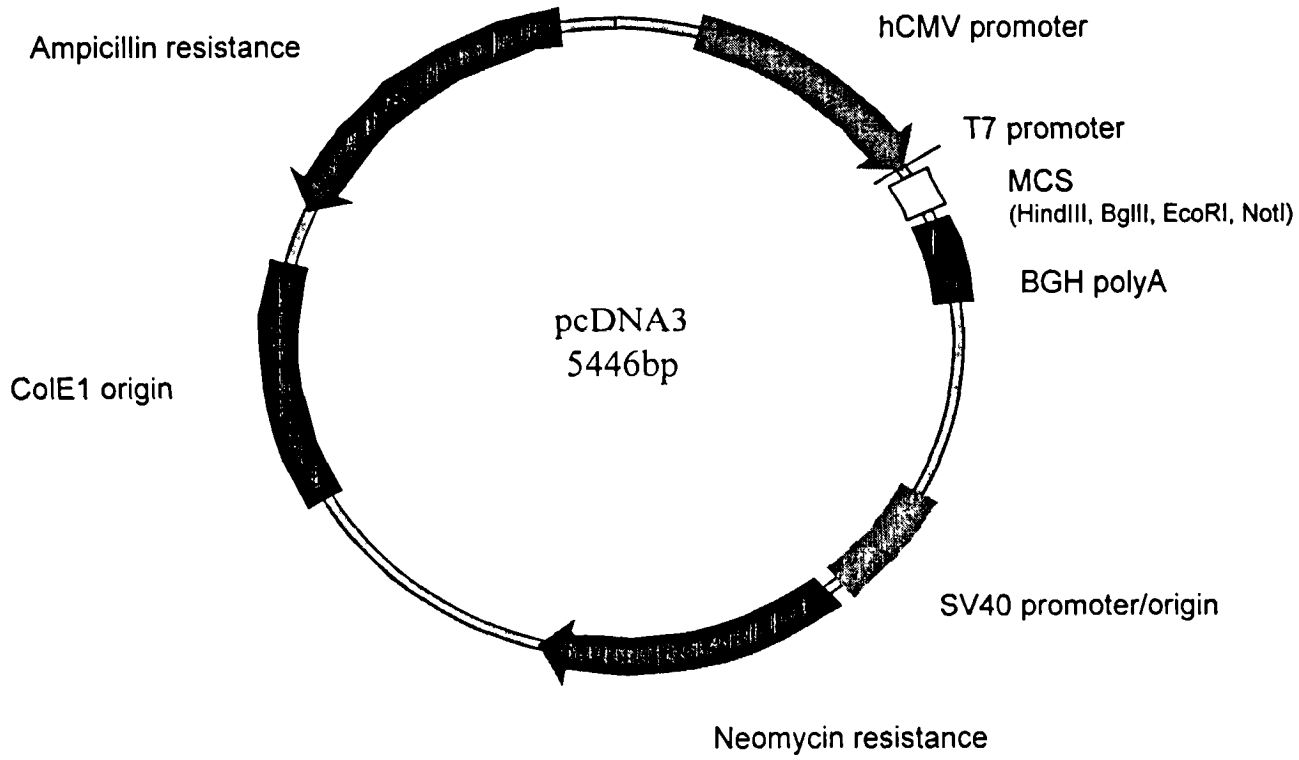


Figure II.3: Schematic map for pEE14.gp120 (P2). This vector was used as the source for the tPA leader sequence, when generating the vector pcDNA3.3p6. The BglII/HindIII fragment identified in pcDNA3.3p6 is indicated by the arrow. The vector was kindly provided by Dr J May.

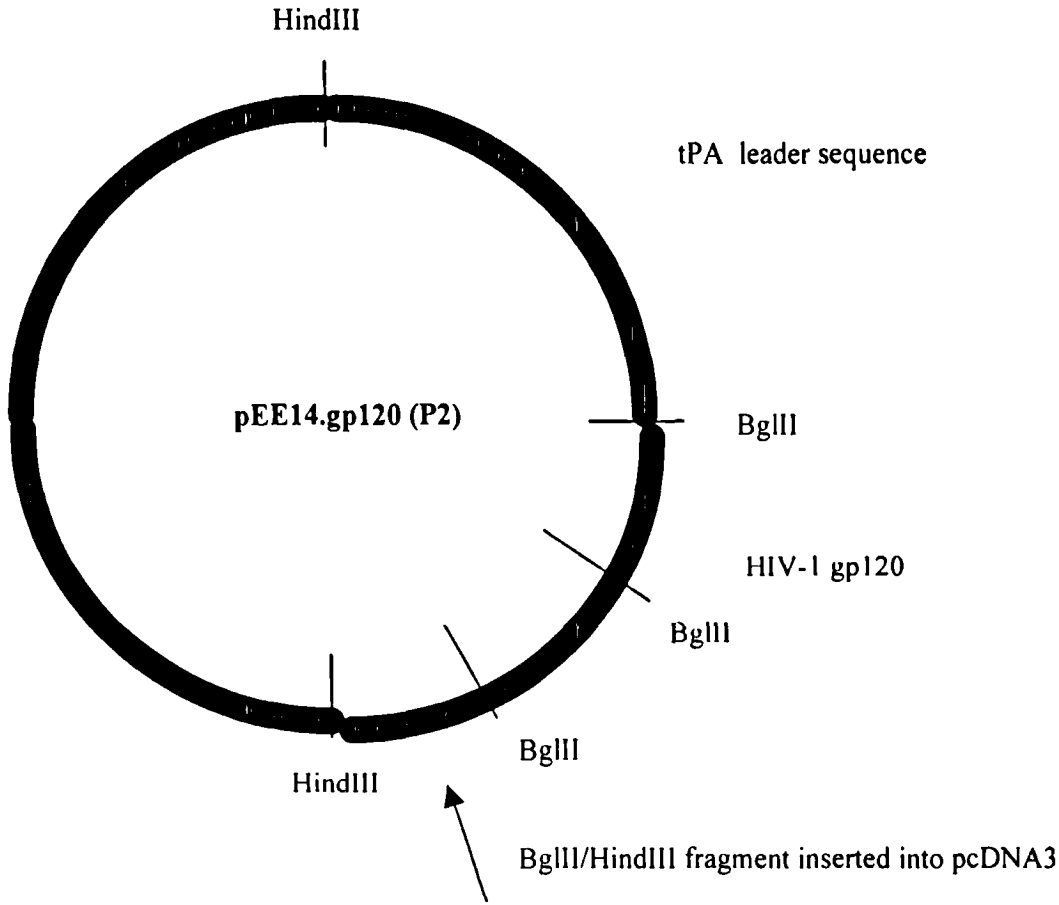


Figure II.3: Schematic map for pcDNA3.3p6tPA. This vector was used for the expression of KSHV glycoprotein sequences with the tPA leader sequence. BGHpolyA: polyadenylation signal and transcription termination sequences from the bovine growth hormone gene; hCMV-MIE: human cytomegalovirus major immediate early enhancer.

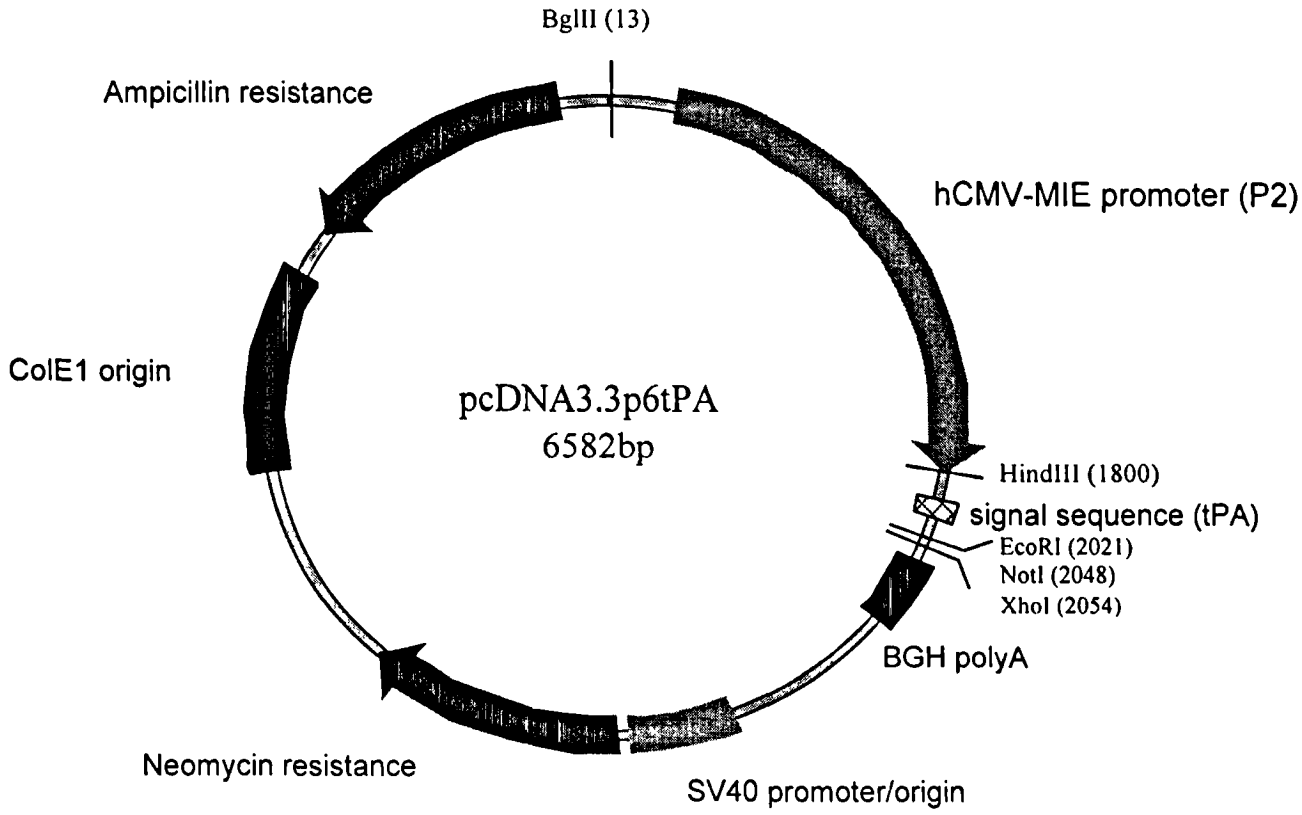


Figure II.4: Schematic map for pcDNA3.1 (Invitrogen). This vector was used for the expression of full length KSHV glycoprotein sequences. MCS: Multiple cloning site; BGHpolyA: polyadenylation signal and transcription termination sequences from the bovine growth hormone gene.

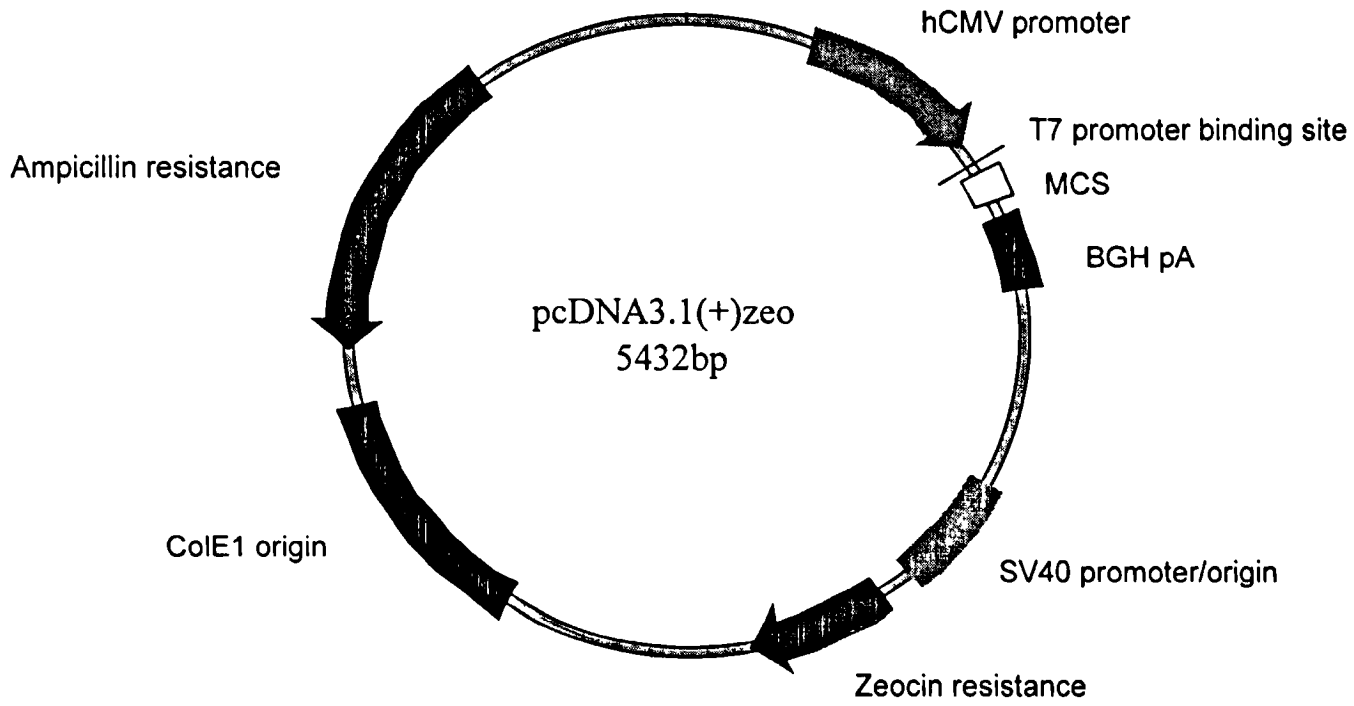


Figure II.5: Schematic map for pcDNA3.1/6xHis/β-gal (Invitrogen). This vector was used to optimise transfection experiments and western blot procedures. BGHpolyA: polyadenylation signal and transcription termination sequences from the bovine growth hormone gene.

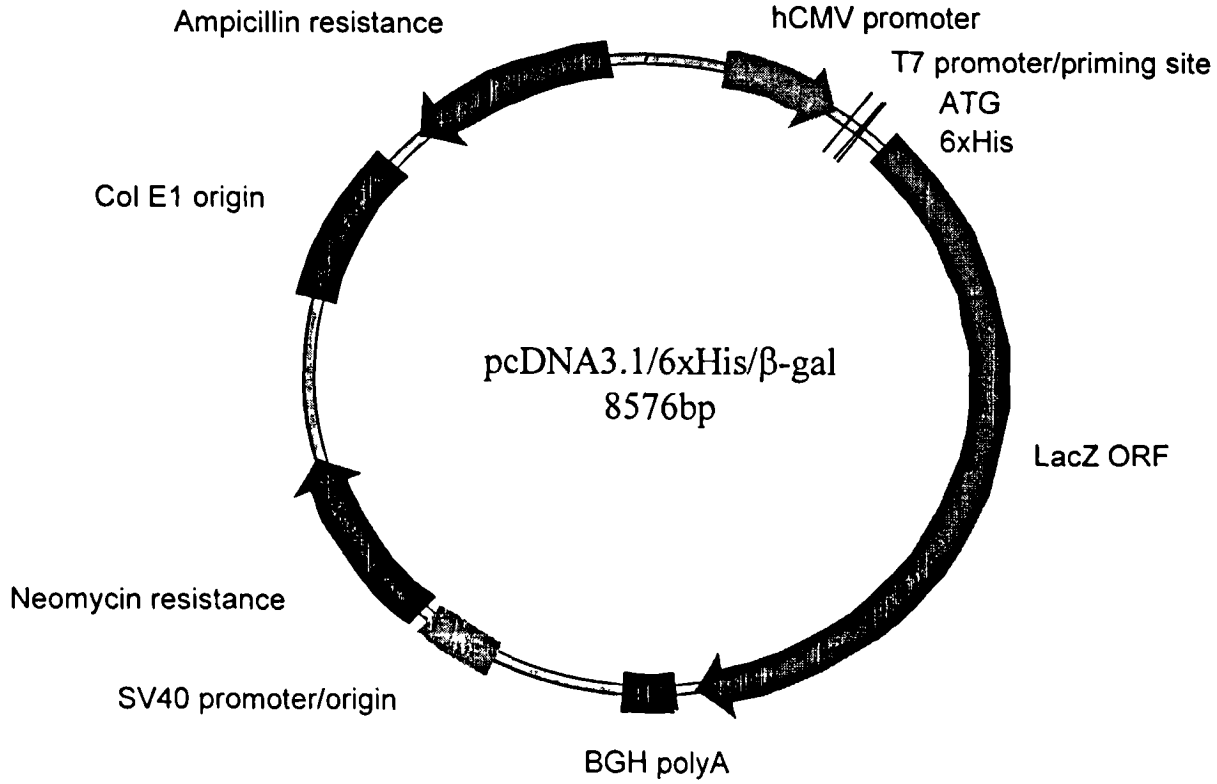
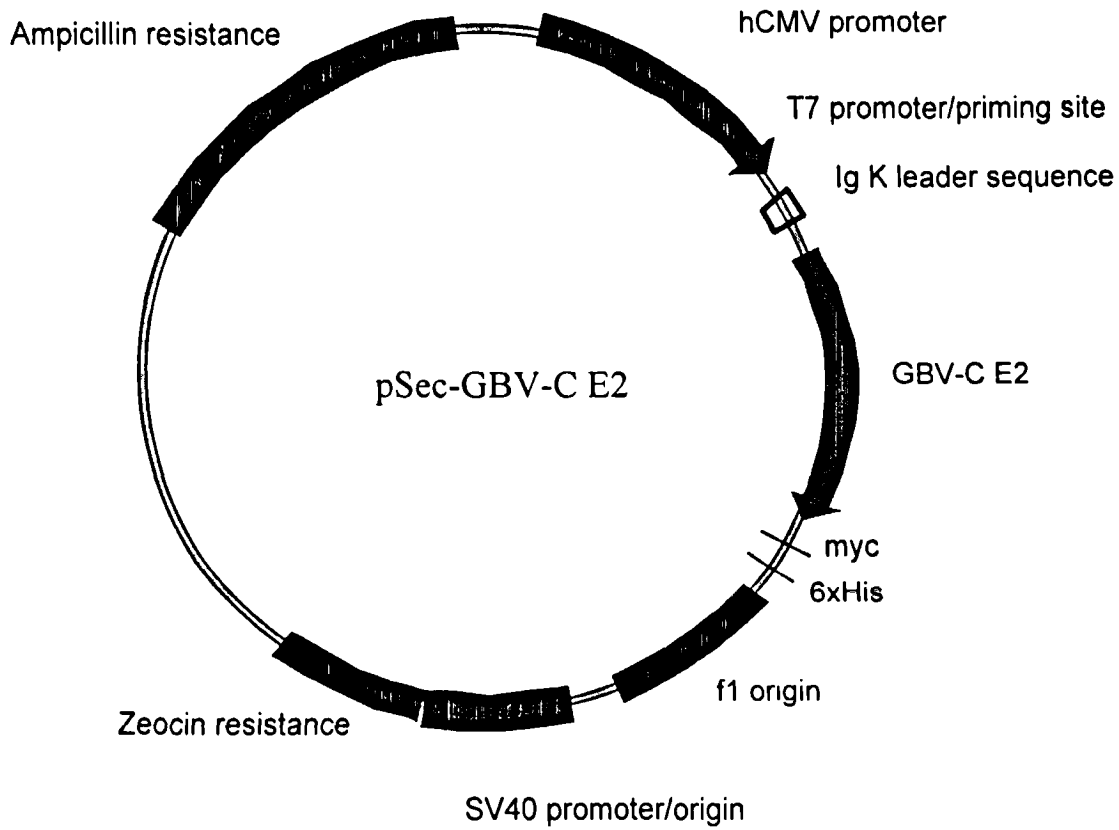


Figure II.6: Schematic map for pSec-GBV-C E2. This vector was used to optimize blue cell assay and western blot procedure. Kindly provided by Dr R Ferns.



Appendix III

Manufacturers' addresses

Advanced Biotechnologies Ltd.	Epsom, UK
Amersham Pharmacia Biotech UK Ltd.	Little Chalfont, UK
Becton Dickinson UK Ltd.	Oxford, UK
Bio-Rad Laboratories Ltd.	Hemel Hempstead, UK
Dako Ltd.	Ely, UK
Harlan Sera-Lab UK Ltd.	Bicester, UK
ICN Pharmaceuticals Ltd.	Basingstoke, UK
Invitrogen BV	Groningen, The Netherlands
Life Technologies Ltd.	Paisley, UK
Merck Ltd.	Leiscester, UK
Molecular Probes Europe BV	Leiden, The Netherlands
Oswel DNA Service	Southampton, UK
Perkin Elmer Biosystems	Warrington, UK
Promega	Southampton, UK
Qiagen Ltd.	Crawley, UK
Roche (Boehringer Mannheim)	Lewes, UK
Sigma-Aldrich Company Ltd.	Poole, UK
Stratagene Ltd.	Cambridge, UK

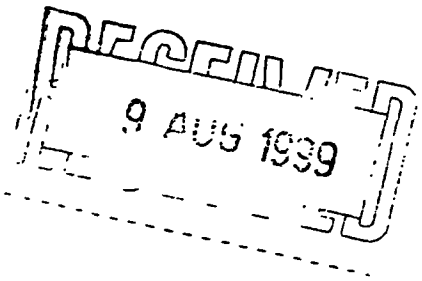
Appendix IV

ST. PETER'S HOSPITAL
(In Association with The Institute of Urology & Nephrology)
THE MIDDLESEX HOSPITAL
Mortimer Street
London W1N 8AA
Telephone: 0171 436 6464
0171 380.....
Fax: 0171 380 9199

DIRECT LINE: 0171 380 9191

FDT.dj

4th August 1999



Professor Richard Tedder
Head of Dept of Virology
Windeyer Building
Cleveland Street

Dear Richard,

Re: Residual patient samples for KSHV DNA study

Thank you for submitting this proposal to the Ethics Committee. I am quite happy for this study to proceed.

Yours sincerely.

Dr F D Thompson
Chairman, Ethics Committee

Royal Free and University College Medical School
UNIVERSITY COLLEGE LONDON

WINDEYER INSTITUTE OF MEDICAL SCIENCES

DEPARTMENT OF VIROLOGY
Windeyer Building
46 Cleveland Street
London W1P 6DB

Telephone 0171 504 9490
Direct Line 0171 504
Fax 0171 580 5896

7 July 1999

Dr F Thompson
Chairman
UCLH Ethics Committee
Department of Uro-Nephrology
Middlesex site

Dear Derek

Re: Residual patient samples for KSHV DNA study

As part of a UCLH Special Trustees-funded PhD studentship, Bianca Elzinger has developed a quantitative assay for KSHV DNA. The assay was initially developed on material derived from KSHV-infected cell-lines maintained in tissue culture. We wish now to investigate the utility of this assay for the detection of cell-free viral DNA present in plasma and in serum. To confirm that both materials will be suitable analytes for analysis, we need to access residual patient materials for testing for KSHV DNA. We intend to use residual material held in the Department on HIV-infected patients where the patient has been contemporaneously bled into a tube for clotted blood and a tube for EDTA anti-coagulated blood. This occurs during the confirmation and final assessment of patients transiting onto anti-retroviral therapy. It will be possible, retrospectively, to identify from our patient/sample files those instances where contemporaneous serum and plasma residual samples are held. We wish to test, randomly and entirely anonymously, pairs of plasma and serum samples for the presence of KSHV DNA. Since this will be undertaken on patients attending consecutively for starting anti-retroviral therapy, and it will be conducted anonymously, this will neither compromise patient management nor lead to patient detriment through inadvertent identification of KSHV infection in an otherwise un-affected individual. The ownership of residual samples on patients is vested in the Trust but for obvious principles of good practice I write to ask your agreement, as Chairman of the Ethical Committee, that the above protocol for access and testing of the samples as laid out as part of Bianca Elzinger's thesis, is appropriate and has your blessing. She approaches the end of her 3rd year with some rapidity, and I would be extremely grateful for a response at your earliest opportunity.

Best wishes

R. Gascon

Richard S Tedder
Head of Department of Virology

RST/eg Dictated and Signed with absence p.p. Tedder

Professor of Medical Virology and Head of Department The Hon Richard S Tedder MA MB BChir MRCP FRCPath